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EDITORIAL

Slow transit constipation: A functional disorder becomes an enteric neuropathy

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Abstract

Slow transit constipation has been traditionally considered and classified as a functional disorder. However, clinical and manometric evidence has been accumulating that suggests how most of the motility alterations in STC might be considered of neuropathic type.In addition, further investigations showed that subtle alterations of the enteric nervous system, not evident to conventional histological examination, may be present in these patients. In the present article we will discuss these evidences, and will try to put them in relation with the abnormal motor function of the large bowel documented in this pathological condition.

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Key words: Constipation; Enteric neuropathy; Functional disorders; Slow transit

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INTRODUCTION

In gastroenterology practice, the most frequently encountered disorders are represented by those related to an abnormal function of the abdominal viscera, the so-called functional diseases[1,2]. The term "functional" defines several variable combinations of chronic or recurrent gastrointestinal symptoms that do not have an identified underlying pathophysiology[3], and that are often also labelled as "idiopathic". Thus, the definition of functional relies on the exclusion of an organic disease, and it is consequently thought to be related to abnormalities of its physiological properties or functions.

Among the so-called functional disorders, one of the most common is functional constipation, whose diagnostic criteria according to Rome Ⅱ classification[4] are reported in Table 1. Patients with functional constipation may be further classified in three subgroups: normal transit constipation, disorders of defecatory or rectal evacuation (outlet obstruction), and slow transit constipation (STC)[5,6]. This latter condition affects mainly women, it is characterized by an often intractable constipation , a heavily delayed colonic transit up to true colonic inertia[7,8], and it is usually attributed to disorders of colonic motor function[9,10]. Indeed, several abnormal motor aspects have been described in STC, such as alterations of rectosigmoid contractile activity[11], decreased colonic propulsive function[12,13], abnormal response to food ingestion[14,15], and overall reduced electrical or motor activity of the large bowel[16,17]. It must also be taken into account that a further cause of delayed colonic transit may be due to the association with pelvic floor dyssynergia; this condition must be identified, since it is amenable of biofeedback treatment, which can normalize colonic transit[18].

However, we still do not know how and why these abnormalities are present in STC patients. In recent years, clinical and manometric evidence has been accumulated that suggests how most of the motility alterations in STC might be considered as a neuropathic type. Moreover, other data showed that some subtle alterations of the enteric nervous system, not evident to conventional histological examination, may be present in these patients. In the present article we will discuss these evidences, and will try to put them in relation with the abnormal motor function of the large bowel documented in this pathological condition.

STC AS A NEUROPATHY: CLINICAL EVIDENCE

In several patients with STC subclinical features of autonomic neuropathy may be present[19], and other studies described selective sensory and autonomic neuropathy in these subjects[20], often with a positive family history, suggesting a genetic basis for this condition[21]. Animal studies might help in elucidating these issues: for instance, transgenic mice with a targeted deletion of neurturin (a neurotrophin) display clinical and tissue phenotype similar to that found in STC, and display associated

Table 1 Rome Ⅱ criteria for functional constipation (adapted from reference 4)

Two or more of the following for at least 12 wk (not necessarily consecutive) in the preceding 12 mo:
• Straining during > 25% of bowel movements; • Lumpy/hard stools for > 25% of bowel movements; • Sensation of incomplete evacuation for > 25% of bowel movements; • Sensation of anorectal blockage for > 25% of bowel movements; • Manual manoeuvres to facilitate > 25% of bowel movements (e.g., digital evacuation or support of the pelvic floor); • < 3 bowel movements per week Loose stools are not present, and there are insufficient criteria for irritable bowel syndrome

parasympathetic deficits[22].

Moreover, since in a sizable proportion of STC patients the symptoms start after pelvic surgical procedures[23-25] or following childbirth[26] it has been hypothesized (although the anatomic proof has never been given) that pelvic nerve injury may occur following hysterectomy and childbirth, and that STC could be considered a disorder of pelvic autonomic nerves at least in a subgroup of patients[27].

STC AS AN ENTERIC NEUROPATHY: INSTRUMENTAL EVIDENCE

Several manometric studies carried out in patients with STC have reported findings that suggest the presence of neuropathic-type abnormalities. Such abnormalities have been described in many instances with respect to the periodic motor activity of the rectosigmoid area (including the so-called rectal motor complex), that appears either decreased or disorganized[28-30], the contractile colonic motor response following intravenous cholinergic stimulation, that in results impaired[31], the early motor response following ingestion of a meal, reported as decreased or absent[32], the overall daily colonic motility, usually decreased to a lesser or greater extent[33,34], the daily organization of regular contractile colonic patterns, that is often impaired[35], and the lack of propulsive response to intraluminal instillation of bisacodyl (a powerful stimulant of mass movements in healthy subjects)[36].

It is worth noting that neuropathic-type abnormalities in patients with STC are not necessarily confined to the large bowel, but may be also documented in other viscera such as the esophagus[37,38], the stomach[39,40], the gallbladder[41], and especially the small bowel[42-45], suggesting the presence of a pan-enteric motor disorder in these patients, particularly in those with more severe symptoms.

STC AS AN ENTERIC NEUROPATHY: PATHOLOGICAL EVIDENCE

Most of the pathologic descriptions related to STC are pertinent to the large bowel, and only sporadic reports from other gastrointestinal viscera (terminal ileum) exist. Therefore, the discussion of pathological findings will focus on the colonic studies.

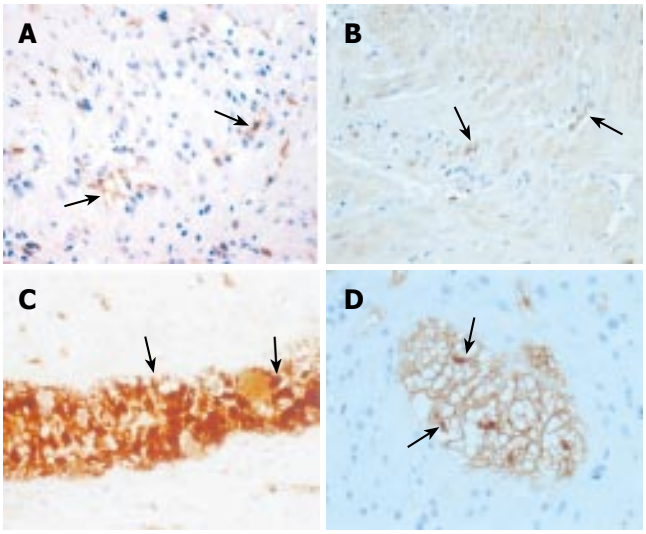

Figure 1 Interstitial cells of Cajal (arrows) at myenteric plexus level in a control subject (**A**) and in a patient with slow transit constipation (**B**). Note the considerable decrease of these cells in the patient (CD117, original magnification x 40). Panels **C** and **D** show the enteric glial cells (arrows) in the myenteric plexus of a control and of a patients with slow transit constipation, respectively. Note the important reduction of these cells in the patient, where only a few ones may be identified (S100, original magnification x 40).

Conventional histology

Most studies employing routine light microscopy have failed to identify consistent abnormalities of the enteric nervous system (ENS) in patients with STC[46-50], apart from the presence of melanosis coli. However, we have recently demonstrated that melanosis coli *per se* does not have any relationship with colonic ENS abnormalities (in particular with the loss of enteric neurons, as hypothesized in older studies only evaluating the submucosa) in these patients[51].

Silver staining technique

Morphological abnormalities of colonic innervation have been described in STC patients using the silver staining technique introduced by Smith[52]. These studies have generally reported a reduction in the total number of argyrophilic neurones and morphological neuronal and/or axonal abnormalities[53,54]. However, the silver staining technique has been subsequently replaced by more modern and reliable immunohistochemical methods (see below).

Immunohistochemistry

Concerning the hypothesized imbalance of enteric neurotransmitters or the enzyme markers (mostly neuropeptides) in STC, the various studies (using immunostaining, immunoassays, or both methods) have frequently yielded inconsistent results. In fact, looking at the findings related to the most commonly investigated neuropeptides (VIP, substance P, neuropeptide Y and 5-HT), decreased, increased or unchanged levels or immunoreactivity has been described in these patients[55-59]. Overall, on the basis of the above reports it might be stated that it is unlikely that alterations of the enteric neurotransmitterns may play

a major role in the pathophysiology of STC. However, more recent observations suggest that an excessive production of nitric oxide in the colonic myenteric plexus of patients with STC could play a pathophysiological role, concurring in the persistent inhibition of contractions[60,61].

More consistent results have been reported with respect to enteric neurons, interstitial cells of Cajal (ICC), and enteric glial cells (EGC). In fact, a decrease of enteric neural elements (neurons and/or neurofilaments) seems to be a constant feature in studies evaluating patients with STC severe enough to require surgery for symptoms' relief[62-66], and these abnormalities are often associated with a reduced number of ICC[67-71], although this latter finding is not constantly present[72]. We have recently shown in a relatively large and homogeneous group of patients with severe and intractable STC, compared to age-matched controls, that the ICC are significantly decreased in patients (Figures 1 A and B), that the enteric neuronal loss may be partially due to apoptotic phenomena, and that these patients display a significantly decreased number of EGC with respect to controls (Figures 1 C and D), in both the submucosal and myenteric plexuses[73].

STC AS AN ENTERIC NEUROPATHY: CLINICAL, INSUMENTAL, AND PATHOLOGICAL RELATIONSHIPS

On the basis of the above evidences, it seems now possible to track a link between the clinical picture, represented by severe constipation with heavily delayed colonic transit, often refractory to medical treatment, the instrumental manometric findings, that mostly show impaired motility and propulsive activity of the large bowel (sometimes with the participation of the upper gastrointestinal segments too), and the abnormalities of the colonic ENS.

The neuronal loss is likely to affect the motor activity of the large bowel, reducing the likelihood of enteric neurotransmission. This defect is then strengthened by the concomitant reduction of the number of ICC, a cell population of paramount importance for the correct homeostasis of gastrointestinal motility. In fact, the primary role of ICC as intestinal pacemakers has been established in experimental animal models, where it has been shown that a lack of ICC networks leads to the absence of slow waves and is accompanied by delayed or absent intestinal motility[74,75]. A decreased ICC function might therefore impair the colonic electrical slow wave activity, thereby affecting the contractile response and causing delayed transit in STC patients. In addition, it has been recently demonstrated that in patients with STC the expression of c-kit mRNA and c-kit protein is significantly decreased compared to controls, suggesting that alterations in the c-kit signal pathway may play an important role in ICC reduction in such patients[76].

An interesting findings, never described before, was the significant decrease of EGC in both the myenteric and submucous plexuses in STC patients compared to controls. This cell population originates from the neural crest and provides both mechanical and physiological support for neuronal elements[77]. The chief known function of the glia in the adult is the formation of myelin sheaths around axons, allowing the fast connections essential for the nervous system function. Moreover, EGC maintain the appropriate concentrations of ions and neurotransmitters in the neuronal environment and are essential regulators of the formation, maintenance and function of synapses, the key functional units of the nervous system[78,79]. Since EGC are thought to act as intermediaries in enteric neurotransmission[80], it is likely that their decrease could synergistically act in further weakening the already precarious neuroenteric balance due to the decrease of neuronal elements and ICC found in patients with STC.

STC AS AN ENTERIC NEUROPATHY: CONCLUSIONS

The case for reclassifying STC other than an "idiopathic" or "functional" disorder is built. In fact, as seen above, clinical, instrumental, and pathological evidences exist that all point toward to a (perhaps) more precise definition of this condition as a true enteric neuropathy. It is probably too early to target STC with a different label, but at least we are now aware of some basic pathophysiological mechanisms potentially responsible for the symptoms and the manometric abnormalities found in this condition. Moreover, apart from mere semantic considerations, the demonstration of such background abnormalities might reveal useful for more targeted therapeutic approaches. For instance, in a mouse model the blockage of Kit receptors caused transdifferentiation of intestinal ICC to a smooth muscle phenotype[81]: if the same would occur in the human colon and if ICC do not die in STC but rather redifferentiate, it might be possible to create conditions that would shift the phenotype back toward ICC.

In conclusion, the advancement of our knowledge of the possible pathophysological mechanisms of "functional" disorders is important for a more correct clinical and therapeutic approach. Further studies are obviously needed before we can drop the "idiopathic" label from these disorders.

REFERENCES

1 **Chang L**. Review article: epidemiology and quality of life in functional gastrointestinal disorders. *Aliment Pharmacol Ther* 2004; **20** Suppl 7: 31-39

2 **Williams M**, Budavari A, Olden KW, Jones MP. Psychosocial assessment of functional gastrointestinal disorders in clinical practice. *J Clin Gastroenterol* 2005; **39**: 847-857

3 **Corazziari E**. Definition and epidemiology of functional gastrointestinal disorders. *Best Pract Res Clin Gastroenterol* 2004; **18**: 613-631

4 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** Suppl 2: II43-II47

5 **Lembo A**, Camilleri M. Chronic constipation. *N Engl J Med* 2003; **349**: 1360-1368

6 **Prather CM**. Subtypes of constipation: sorting out the confusion. *Rev Gastroenterol Disord* 2004; **4** Suppl 2: S11-S16

7 **Knowles CH**, Martin JE. Slow transit constipation: a model of human gut dysmotility. Review of possible aetiologies. *Neurogastroenterol Motil* 2000; **12**: 181-196

8 **Bassotti G**, Roberto GD, Sediari L, Morelli A. Toward a defini-

tion of colonic inertia. *World J Gastroenterol* 2004; **10**: 2465-2467
9 **Bharucha AE**, Phillips SF. Slow transit constipation. *Gastroenterol Clin North Am* 2001; **30**: 77-95
10 **Bassotti G**, de Roberto G, Castellani D, Sediari L, Morelli A. Normal aspects of colorectal motility and abnormalities in slow transit constipation. *World J Gastroenterol* 2005; **11**: 2691-2696
11 **Preston DM**, Lennard-Jones JE. Pelvic motility and response to intraluminal bisacodyl in slow-transit constipation. *Dig Dis Sci* 1985; **30**: 289-294
12 **Kamm MA**, van der Sijp JR, Lennard-Jones JE. Observations on the characteristics of stimulated defaecation in severe idiopathic constipation. *Int J Colorectal Dis* 1992; **7**: 197-201
13 **Bassotti G**, Chistolini F, Nzepa FS, Morelli A. Colonic propulsive impairment in intractable slow-transit constipation. *Arch Surg* 2003; **138**: 1302-1304
14 **Bassotti G**, Imbimbo BP, Betti C, Dozzini G, Morelli A. Impaired colonic motor response to eating in patients with slow-transit constipation. *Am J Gastroenterol* 1992; **87**: 504-508
15 **Björnsson ES**, Chey WD, Hooper F, Woods ML, Owyang C, Hasler WL. Impaired gastrocolonic response and peristaltic reflex in slow-transit constipation: role of 5-HT(3) pathways. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G400-G407
16 **Schang JC**, Devroede G, Duguay C, Hémond M, Hébert M. [Constipation caused by colonic inertia and distal obstruction: electromyographic study]. *Gastroenterol Clin Biol* 1985; **9**: 480-485
17 **Bassotti G**, Gaburri M, Imbimbo BP, Rossi L, Farroni F, Pelli MA, Morelli A. Colonic mass movements in idiopathic chronic constipation. *Gut* 1988; **29**: 1173-1179
18 **Chiarioni G**, Salandini L, Whitehead WE. Biofeedback benefits only patients with outlet dysfunction, not patients with isolated slow transit constipation. *Gastroenterology* 2005; **129**: 86-97
19 **Altomare D**, Pilot MA, Scott M, Williams N, Rubino M, Ilincic L, Waldron D. Detection of subclinical autonomic neuropathy in constipated patients using a sweat test. *Gut* 1992; **33**: 1539-1543
20 **Raethjen J**, Pilot MA, Knowles C, Warner G, Anand P, Williams N. Selective autonomic and sensory deficits in slow transit constipation. *J Auton Nerv Syst* 1997; **66**: 46-52
21 **Knowles CH**, Scott SM, Wellmer A, Misra VP, Pilot MA, Williams NS, Anand P. Sensory and autonomic neuropathy in patients with idiopathic slow-transit constipation. *Br J Surg* 1999; **86**: 54-60
22 **Heuckeroth RO**, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EM Jr, Milbrandt J. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* 1999; **22**: 253-263
23 **Roe AM**, Bartolo DC, Mortensen NJ. Slow transit constipation. Comparison between patients with or without previous hysterectomy. *Dig Dis Sci* 1988; **33**: 1159-1163
24 **Vierhout ME**, Schreuder HW, Veen HF. Severe slow-transit constipation following radical hysterectomy. *Gynecol Oncol* 1993; **51**: 401-403
25 **MacDonald A**, Baxter JN, Finlay IG. Idiopathic slow-transit constipation. *Br J Surg* 1993; **80**: 1107-1111
26 **MacDonald A**, Baxter JN, Bessent RG, Gray HW, Finlay IG. Gastric emptying in patients with constipation following childbirth and due to idiopathic slow transit. *Br J Surg* 1997; **84**: 1141-1143
27 **Knowles CH**, Scott SM, Lunniss PJ. Slow transit constipation: a disorder of pelvic autonomic nerves? *Dig Dis Sci* 2001; **46**: 389-401
28 **Waldron DJ**, Kumar D, Hallan RI, Wingate DL, Williams NS. Evidence for motor neuropathy and reduced filling of the rectum in chronic intractable constipation. *Gut* 1990; **31**: 1284-1288
29 **Bassotti G**, Betti C, Pelli MA, Morelli A. Prolonged (24-hour) manometric recording of rectal contractile activity in patients with slow transient constipation. *Digestion* 1991; **49**: 72-77
30 **Rao SS**, Sadeghi P, Batterson K, Beaty J. Altered periodic rectal motor activity: a mechanism for slow transit constipation. *Neurogastroenterol Motil* 2001; **13**: 591-598
31 **Bassotti G**, Chiarioni G, Imbimbo BP, Betti C, Bonfante F, Vantini I, Morelli A, Whitehead WE. Impaired colonic motor response to cholinergic stimulation in patients with severe chronic idiopathic (slow transit type) constipation. *Dig Dis Sci* 1993; **38**: 1040-1045
32 **Bassotti G**, Morelli A, Whitehead WE. Abnormal rectosigmoid myoelectric response to eating in patients with severe idiopathic constipation (slow-transit type). *Dis Colon Rectum* 1992; **35**: 753-756
33 **Rao SS**, Sadeghi P, Beaty J, Kavlock R. Ambulatory 24-hour colonic manometry in slow-transit constipation. *Am J Gastroenterol* 2004; **99**: 2405-2416
34 **Shafik A**, Shafik AA, El-Sibai O, Mostafa RM. Electric activity of the colon in subjects with constipation due to total colonic inertia: an electrophysiologic study. *Arch Surg* 2003; **138**: 1007-1011; discussion 1011
35 **Bassotti G**, Chistolini F, Battaglia E, Chiarioni G, Nzepa FS, Dughera L, deRoberto G, Emanuelli G, Morelli A. Are colonic regular contractile frequency patterns in slow transit constipation a relevant pathophysiological phenomenon? *Dig Liver Dis* 2003; **35**: 552-556
36 **Bassotti G**, Chiarioni G, Germani U, Battaglia E, Vantini I, Morelli A. Endoluminal instillation of bisacodyl in patients with severe (slow transit type) constipation is useful to test residual colonic propulsive activity. *Digestion* 1999; **60**: 69-73
37 **Watier A**, Devroede G, Duranceau A, Abdel-Rahman M, Duguay C, Forand MD, Tétreault L, Arhan P, Lamarche J, Elhilali M. Constipation with colonic inertia. A manifestation of systemic disease? *Dig Dis Sci* 1983; **28**: 1025-1033
38 **Altomare DF**, Portincasa P, Rinaldi M, Di Ciaula A, Martinelli E, Amoruso A, Palasciano G, Memeo V. Slow-transit constipation: solitary symptom of a systemic gastrointestinal disease. *Dis Colon Rectum* 1999; **42**: 231-240
39 **van der Sijp JR**, Kamm MA, Nightingale JM, Britton KE, Granowska M, Mather SJ, Akkermans LM, Lennard-Jones JE. Disturbed gastric and small bowel transit in severe idiopathic constipation. *Dig Dis Sci* 1993; **38**: 837-844
40 **Mollen RM**, Hopman WP, Oyen WJ, Kuijpers HH, Edelbroek MA, Jansen JB. Effect of subtotal colectomy on gastric emptying of a solid meal in slow-transit constipation. *Dis Colon Rectum* 2001; **44**: 1189-1195
41 **Gunay A**, Gurbuz AK, Narin Y, Ozel AM, Yazgan Y. Gallbladder and gastric motility in patients with idiopathic slow-transit constipation. *South Med J* 2004; **97**: 124-128
42 **Bassotti G**, Stanghellini V, Chiarioni G, Germani U, De Giorgio R, Vantini I, Morelli A, Corinaldesi R. Upper gastrointestinal motor activity in patients with slow-transit constipation. Further evidence for an enteric neuropathy. *Dig Dis Sci* 1996; **41**: 1999-2005
43 **Glia A**, Lindberg G. Antroduodenal manometry findings in patients with slow-transit constipation. *Scand J Gastroenterol* 1998; **33**: 55-62
44 **Scott SM**, Picon L, Knowles CH, Fourquet F, Yazaki E, Williams NS, Lunniss PJ, Wingate DL. Automated quantitative analysis of nocturnal jejunal motor activity identifies abnormalities in individuals and subgroups of patients with slow transit constipation. *Am J Gastroenterol* 2003; **98**: 1123-1134
45 **Panagamuwa B**, Kumar D, Ortiz J, Keighley MR. Motor abnormalities in the terminal ileum of patients with chronic idiopathic constipation. *Br J Surg* 1994; **81**: 1685-1688
46 **Preston DM**, Hawley PR, Lennard-Jones JE, Todd IP. Results of colectomy for severe idiopathic constipation in women (Arbuthnot Lane's disease). *Br J Surg* 1984; **71**: 547-552
47 **Kamm MA**, Hawley PR, Lennard-Jones JE. Outcome of colectomy for severe idiopathic constipation. *Gut* 1988; **29**: 969-973
48 **Walsh PV**, Peebles-Brown DA, Watkinson G. Colectomy for slow transit constipation. *Ann R Coll Surg Engl* 1987; **69**: 71-75
49 **Bassotti G**, Betti C, Pelli MA, Morelli A. Extensive investigation on colonic motility with pharmacological testing is useful for selecting surgical options in patients with inertia colica. *Am J Gastroenterol* 1992; **87**: 143-147

50 **Redmond JM**, Smith GW, Barofsky I, Ratych RE, Goldsborough DC, Schuster MM. Physiological tests to predict long-term outcome of total abdominal colectomy for intractable constipation. *Am J Gastroenterol* 1995; **90**: 748-753

51 **Villanacci V**, Bassotti G, Cathomas G, Maurer CA, Di Fabio F, Fisogni S, Cadei M, Mazzocchi A, Salerni B. Is pseudomelanosis coli a marker of colonic neuropathy in severely constipated patients? *Histopathology* 2006; **49**: 132-137

52 **Smith B**. Myenteric plexus in Hirschsprung's disease. *Gut* 1967; **8**: 308-312

53 **Krishnamurthy S**, Schuffler MD, Rohrmann CA, Pope CE 2nd. Severe idiopathic constipation is associated with a distinctive abnormality of the colonic myenteric plexus. *Gastroenterology* 1985; **88**: 26-34

54 **Zenilman ME**, Dunnegan DL, Soper NJ, Becker JM. Successful surgical treatment of idiopathic colonic dysmotility. The role of preoperative evaluation of coloanal motor function. *Arch Surg* 1989; **124**: 947-951

55 **Koch TR**, Carney JA, Go L, Go VL. Idiopathic chronic constipation is associated with decreased colonic vasoactive intestinal peptide. *Gastroenterology* 1988; **94**: 300-310

56 **Dolk A**, Brodén G, Holmström B, Johansson C, Schultzberg M. Slow transit chronic constipation (Arbuthnot Lane's disease). An immunohistochemical study of neuropeptide-containing nerves in resected specimens from the large bowel. *Int J Colorectal Dis* 1990; **5**: 181-187

57 **Lincoln J**, Crowe R, Kamm MA, Burnstock G, Lennard-Jones JE. Serotonin and 5-hydroxyindoleacetic acid are increased in the sigmoid colon in severe idiopathic constipation. *Gastroenterology* 1990; **98**: 1219-1225

58 **Tzavella K**, Riepl RL, Klauser AG, Voderholzer WA, Schindlbeck NE, Müller-Lissner SA. Decreased substance P levels in rectal biopsies from patients with slow transit constipation. *Eur J Gastroenterol Hepatol* 1996; **8**: 1207-1211

59 **Sjölund K,** Fasth S, Ekman R, Hultén L, Jiborn H, Nordgren S, Sundler F. Neuropeptides in idiopathic chronic constipation (slow transit constipation). *Neurogastroenterol Motil* 1997; **9**: 143-150

60 **Cortesini C**, Cianchi F, Infantino A, Lise M. Nitric oxide synthase and VIP distribution in enteric nervous system in idiopathic chronic constipation. *Dig Dis Sci* 1995; **40**: 2450-2455

61 **Tomita R**, Fujisaki S, Ikeda T, Fukuzawa M. Role of nitric oxide in the colon of patients with slow-transit constipation. *Dis Colon Rectum* 2002; **45**: 593-600

62 **Schouten WR**, ten Kate FJ, de Graaf EJ, Gilberts EC, Simons JL, Klück P. Visceral neuropathy in slow transit constipation: an immunohistochemical investigation with monoclonal antibodies against neurofilament. *Dis Colon Rectum* 1993; **36**: 1112-1117

63 **Park HJ**, Kamm MA, Abbasi AM, Talbot IC. Immunohistochemical study of the colonic muscle and innervation in idiopathic chronic constipation. *Dis Colon Rectum* 1995; **38**: 509-513

64 **Porter AJ**, Wattchow DA, Hunter A, Costa M. Abnormalities of nerve fibers in the circular muscle of patients with slow transit constipation. *Int J Colorectal Dis* 1998; **13**: 208-216

65 **Faussone-Pellegrini MS**, Infantino A, Matini P, Masin A, Mayer B, Lise M. Neuronal anomalies and normal muscle morphology at the hypomotile ileocecocolonic region of patients affected by idiopathic chronic constipation. *Histol Histopathol* 1999; **14**: 1119-1134

66 **Wedel T**, Roblick UJ, Ott V, Eggers R, Schiedeck TH, Krammer HJ, Bruch HP. Oligoneuronal hypoganglionosis in patients with idiopathic slow-transit constipation. *Dis Colon Rectum* 2002; **45**: 54-62

67 **He CL**, Burgart L, Wang L, Pemberton J, Young-Fadok T, Szurszewski J, Farrugia G. Decreased interstitial cell of cajal volume in patients with slow-transit constipation. *Gastroenterology* 2000; **118**: 14-21

68 **Lyford GL**, He CL, Soffer E, Hull TL, Strong SA, Senagore AJ, Burgart LJ, Young-Fadok T, Szurszewski JH, Farrugia G. Pancolonic decrease in interstitial cells of Cajal in patients with slow transit constipation. *Gut* 2002; **51**: 496-501

69 **Wedel T**, Spiegler J, Soellner S, Roblick UJ, Schiedeck TH, Bruch HP, Krammer HJ. Enteric nerves and interstitial cells of Cajal are altered in patients with slow-transit constipation and megacolon. *Gastroenterology* 2002; **123**: 1459-1467

70 **Tong WD**, Liu BH, Zhang LY, Zhang SB, Lei Y. Decreased interstitial cells of Cajal in the sigmoid colon of patients with slow transit constipation. *Int J Colorectal Dis* 2004; **19**: 467-473

71 **Lee JI**, Park H, Kamm MA, Talbot IC. Decreased density of interstitial cells of Cajal and neuronal cells in patients with slow-transit constipation and acquired megacolon. *J Gastroenterol Hepatol* 2005; **20**: 1292-1298

72 **Toman J**, Turina M, Ray M, Petras RE, Stromberg AJ, Galandiuk S. Slow transit colon constipation is not related to the number of interstitial cells of Cajal. *Int J Colorectal Dis* 2006; **21**: 527-532

73 **Bassotti G**, Villanacci V, Maurer CA, Fisogni S, Di Fabio F, Cadei M, Morelli A, Panagiotis T, Cathomas G, Salerni B. The role of glial cells and apoptosis of enteric neurones in the neuropathology of intractable slow transit constipation. *Gut* 2006; **55**: 41-46

74 **Ward SM**, Burns AJ, Torihashi S, Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *J Physiol* 1994; **480** (Pt 1): 91-97

75 **Huizinga JD**, Thuneberg L, Klüppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* 1995; **373**: 347-349

76 **Tong WD**, Liu BH, Zhang LY, Xiong RP, Liu P, Zhang SB. Expression of c-kit messenger ribonucleic acid and c-kit protein in sigmoid colon of patients with slow transit constipation. *Int J Colorectal Dis* 2005; **20**: 363-367

77 **Rühl A**. Glial cells in the gut. *Neurogastroenterol Motil* 2005; **17**: 777-790

78 **Gershon MD**, Rothman TP. Enteric glia. *Glia* 1991; **4**: 195-204

79 **Jessen KR**. Glial cells. *Int J Biochem Cell Biol* 2004; **36**: 1861-1867

80 **Rühl A**, Nasser Y, Sharkey KA. Enteric glia. *Neurogastroenterol Motil* 2004; **16** Suppl 1: 44-49

81 **Torihashi S**, Nishi K, Tokutomi Y, Nishi T, Ward S, Sanders KM. Blockade of kit signaling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. *Gastroenterology* 1999; **117**: 140-148

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REVIEW

Intestinal mucosal adaptation

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Abstract

Intestinal failure is a condition characterized by malnutrition and/or dehydration as a result of the inadequate digestion and absorption of nutrients. The most common cause of intestinal failure is short bowel syndrome, which occurs when the functional gut mass is reduced below the level necessary for adequate nutrient and water absorption. This condition may be congenital, or may be acquired as a result of a massive resection of the small bowel. Following resection, the intestine is capable of adaptation in response to enteral nutrients as well as other trophic stimuli. Identifying factors that may enhance the process of intestinal adaptation is an exciting area of research with important potential clinical applications.

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Key words: Small intestine; Transport; Morphology; Resection; Short bowel syndrome; Absorption; Diet; Gene expression; Hyperplasia; Enterocytes

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STRUCTURAL AND FUNCTIONAL ADAPTATION

The intestine has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli. In fact, intestinal adaptation may be considered as a paradigm of gene-environment interactions. The array of phenotypic adaptations includes the modification of brush border membrane (BBM) fluidity and permeability, as well as up- or down-regulation of carrier-mediated transport. In animal models, intestinal adaptation occurs following the loss of a major portion of the small intestine ("short bowel syndrome", SBS), following chronic ingestion of ethanol, sublethal doses of abdominal irradiation, diabetes, aging, fasting and malnutrition[1-5]. Following intestinal resection, morphological and functional changes occur depending upon the extent of the intestine removed, the site studied, and the lipid content of the diet[6]. The increase in nutrient absorption compensates for the loss of absorptive surface area, and minimizes the malabsorption that could otherwise potentially occur. Therefore, intestinal adaptation has important implications in the survival potential and welfare of the host[7]. However, in some cases such as diabetes, intestinal adaptation may have deleterious effects, with enhanced nutrient uptake exacerbating prevailing hyperglycemia, hyperlipidemia and obesity[8].

The mechanisms of intestinal adaptation occur at a variety of levels: physiological, cellular and molecular. Signals of adaptation may relate to various hormone levels, transcription factors, ATP levels, or changes in the concentration of luminal solutes[5]. The signals and mechanisms of the adaptive process may be different for the jejunum and ileum, as well as in the intestinal crypt and villous tip, explaining the site-specific alterations and differences between crypt and villous enterocytes[3,4].

Rodents are commonly used in well-characterized models of assessing the process of intestinal adaptation[9]. Following small bowel resection in the rat, the remnant intestinal mucosa undergoes compensatory alterations in an attempt to restore normal absorptive capacity[10]. Morphologic and functional changes include increases in crypt depth and villous length, enterocyte proliferation, as well as increased electrolyte, glucose and amino acid uptake[9,10].

The adaptive process has been defined in terms of transport kinetics. Changes usually occur in the value of the maximal transport rate (Vmax) rather than in the Michaelis affinity (Km) constant of specific nutrient transporters (sugars and amino acids)[11,12]. Furthermore, there may be alterations in the passive permeability coefficients of nutrients transported passively such as short-, medium- and long chain fatty acids and cholesterol[3,4,13]. The increased Vmax results from either an up-regulation of the total number of transporters, an increased number of transporting mucosal cells, or an increase in the intrinsic activity fo the transporter[14,15]. Intestinal resection also selectively changes the passive permeability properties of the BBM, as demonstrated by the increased uptake of fatty acids following intestinal resection, an increase that was not due to the changes in the mucosal surface area or the effective resistance of

the intestinal unstirred water layer (UWL)[16]. Indeed, this altered permeability is due to changes in the lipophilic properties of the BBM due to variations in the lipid content of the BBM[17].

Intestinal adaptation in the rodent model of chronic diabetes involves changes at the transcriptional as well as the posttranscriptional level, leading to increased Na^+-coupled sugar absorption[18]. After inducing acute hyperglycemia in rats, there is rapid up-regulation of glucose transport across the basolateral membrane (BLM) of the enterocytes[19]. In this model, both the vascular as well as luminal glucose infusion causes an increase in the glucose transport capacity across the BLM[20]. However, no significant increase in BLM cytochalasin B binding or in GLUT2 protein abundance was observed, suggesting that there may be a post-translational event that increases the number of GLUT2 proteins available for transport, such as the movement of GLUT2 to the BLM from a preformed pool within the enterocyte. Alternatively, the "intrinsic activity" of the transporter may be altered in the absence of changes in the protein abundance. Changes in the intrinsic activity of glucose transporters have been observed with hyperglycemia[21], diabetes[22], low luminal glucose concentrations[14] and following the activation of MAPK and PI3K[15].

Following extensive intestinal resection, there is hyperplasia of the remaining bowel, which may be accompanied by the enhanced uptake of nutrients[23]. The alterations in the cell kinetics that result in modification of the nutrition status may be specific or non-specific. *Non-specific mechanisms* involve alterations that result in changes in the intestinal mucosal mass and/or the villous surface area, leading to modifications in the uptake of all nutrients, including those that are absorbed passively[24]. On the other hand, *specific mechanisms* involve up- or down-regulation of transporters responsible for the uptake of particular nutrients, such as sugars or amino acids[3,4].

The observation that morphological modifications may accompany intestinal adaptation in the rodent small bowel resection model was first made by Dowling and Booth[23]. The remaining intestine after resection is hyperplastic, with greater villous height and crypt depth, leading to enhance mucosal surface area. However, while enhanced nutrient absorption is observed, the morphological changes do not necessarily explain the alterations in nutrient uptake. For example, one week after an 80% small bowel resection, the remaining intestine increased its mass to 50%-70% of its pre-resection level, yet the uptake of glucose increased only to approximately 33% of the pre-resection level[10]. Thus, enhanced nutrient absorption may not be solely explained by intestinal hyperplasia and/or hypertrophy.

It is clear that dynamic morphologic parameters of the intestine may also adapt. For instance, the crypt cell production rates or the enterocyte migration rates change in some situations of intestinal adaptation[25]. It is important that morphological alterations are considered when estimating the kinetic parameters of absorption. Morphological modifications such as blunting of the mucosal growth or mucosal hyperplasia after intestinal resection are observed when Dexamethasone (Dex) is given subcutaneously[26]. Both kinetics and dynamic morphologic parameters are altered in the adaptive process, and the influence of resection on nutrient uptake is due in part to these kinetic alterations. This may be due to the altered cell kinetics changing the population of the enterocytes along the villus, thereby leading to variations in the number of cells with transporter, or the activity of the transporters[27,28].

Many animal models of intestinal adaptation have been described: glucose uptake has been found to be increased during pregnancy[24], lactation[30], with the ingestion of a high carbohydrate diet[31], hyperglycemia[32], with diabetes[33], high alcohol intake[34] and after intestinal resection[35]. On the other hand, glucose uptake is decreased with aging[36], external abdominal radiation[37] and with the use of total parenteral nutrition[38]. Most transporters are up-regulated by the levels of dietary substrate levels, whereas toxic substances and essential amino acids have the opposite effect[9,38-40]. These examples illustrate the diversity and variability of this intestinal adaptive process.

The adaptive response in humans is not well characterized. Increases in nutrient absorption have been documented[41-43] in humans following resection. The role of morphological changes in this process, however, has not been conclusively demonstrated. Remnant small bowel lengthening and dilatation has been noted in patients with SBS, suggesting morphologic mechanisms in human intestinal adaptation[44]. However, the mucosal adaptation typical in rodent models is not seen in the human adaptive response[45,46]. Indeed, several studies have shown that no increases in villous height or crypt depth were detected among patients who underwent intestinal resection, as compared to healthy controls[42,47].

With the existence of various relevant anatomical, physiological and biochemical differences between the human and rodent gastrointestinal tracts[48], and a conspicuous lack of comparable human studies, the clinical adequacy of the rat as a model of intestinal adaptation remains to be determined. Although the morphological and functional changes that occur in the rodent following massive small bowel resection have been well characterized[23,49], direct evidence for similar changes in humans is lacking. Accordingly, caution must be used when attempting to extrapolate findings from rodent studies to the human population. An alternative model, the neonatal piglet, has been used in short bowel studies[50-52]. The neonatal pig has recently been used to determine the effects of IGF-1 and dietary manipulations in an intestinal resection model[53,54]. The degree to which the results obtained using this model reflect human findings has yet to be determined, and the rodent remains a popular model for studies of intestinal adaptation.

DIETARY REGULATION

The topic of the dietary regulation of intestinal gene expression has been reviewed[31,55]. Dietary constituents provide continual environmental signals that elicit the expression of a host of genes that influence intestinal adaptation[56]. Every day, enterocytes are exposed to different nutrients that vary according to the nutrient intake of the host. For this reason, the intestine must be able to

adapt to variations in the dietary load and composition[31,57]. The intestine, like many other biological and engineered systems, is quantitatively matched to prevailing peak loads with modest reserve capacities. Indeed, physiological capacities are optimal and most economical if they ascribe to the adage "enough, but not too much"[57]. Therefore, intestinal enzymes and transporters are characterized by a "safety factor", a parameter that represents the ratio of its capacity to the load placed on it[58]. The maintenance of this reserve capacity is biosynthetically costly, but is necessary given the unpredictable nature of dietary contents.

Parenteral vs enteral nutrition

Small bowel atrophy is well characterized in rodent models using total parenteral nutrition[59-61]. Not surprisingly, the presence of luminal nutrients also contributes greatly to the adaptive process. Intestinal adaptation following massive small bowel resection is limited, but not entirely abolished in the absence of luminal nutrition[62]. The following sections detail the effects of the type and amount of various luminal nutrients on the adaptive process.

Lipids

Dietary fat content influences the uptake of hexoses and lipids into rabbit jejunum following ileal resection[16]. More recently, using a rat model of SBS, Sukhotnik *et al* (2003) demonstrated that early feeding of a high fat diet increased lipid absorptive capacity of the intestinal remnant[63]. The main mechanisms of this effect may be an acceleration of structural intestinal adaptation, resulting in an increased number of enterocytes. However, at the molecular and cellular level, a high fat diet decreased mucosal mRNA levels of the lipid binding protein FAT/CD36 and decreased oleic acid uptake by isolated enterocytes. This is in contrast to what is seen with the liver fatty acid binding protein (L-FABP), a cytosolic lipid binding protein. Mice that were chronically fed a diet enriched in sunflower oil had increased the liver fatty acid binding protein (L-FABP) mRNA levels in their small intestine[64]. The effect was specific to this gene, as the intestinal fatty acid binding protein (I-FABP) was unaffected.

Not only the amount of fat, but also the type of dietary fat may influence intestinal function. Keelan *et al* (1996) tested the hypothesis that the intestinal morphology and uptake of nutrients after resection of the distal half of the small intestine in rats responds to alterations in the dietary content of saturated (SFA) and polyunsaturated (PUFA) fatty acids[65]. Adult female Sprague-Dawley rats were subjected to a sham operation or to the surgical resection of the distal half of the small intestine. The animals were fed chow for 3 wk, then either chow or isocaloric semisynthetic SFA or PUFA diets for a further 2 wk. The *in vitro* jejunal uptake of glucose was twice as high in animals that had undergone resection and were fed SFA than in those fed PUFA. It was suggested that SFA was necessary in the diet to ensure that adequate adaptation takes place.

Thiesen and colleagues examined the effect of dietary lipids on lipid uptake in rats post-resection. Intestinal resection had no effect on the mRNA expression of early response genes (ERGs), proglucagon, or the ileal lipid binding protein (ILBP), but was associated with reduced jejunal mRNA for ornithine decarboxylase (ODC) and for the liver fatty acid binding protein (L-FABP)[66]. These resection-associated changes in gene expression were not linked with alterations in the intestinal uptake of long chain fatty acids or cholesterol. In animals undergoing intestinal resection and fed SFA or given control vehicle, there was a reduction in jejunal proglucagon mRNA expression as compared to those animals fed chow or PUFA. ODC mRNA expression in the jejunum of resected animals was reduced. Thus, dietary lipids modify the uptake of lipids in resected animals, and ODC and proglucagon may be involved in this adaptive response[67].

The way by which dietary lipids alter gene expression and consequently change membrane composition and/or nutrient transport may be through the activation of peroxisome proliferator-activated receptors (PPAR), hepatic nuclear factor-4 (HNF-4), nuclear factor κ B (NFκB), and sterol response element binding proteins 1c (SREBP1c)[56]. By binding to these transcriptional factors, dietary lipids affect the rate of transcription and consequently the protein synthesis of nutrient transporters[65,68]. It is also known that PPARs belong to the superfamily of receptors that include the glucocorticosteroid receptor (GR)[69]. When the locally acting glucocorticosteroid (GC) budesonide was administered concomitantly with SFA diet, the jejunal uptake of glucose was increased but the ileal uptake of fructose was reduced[70].

It has been suggested that dietary lipids participate in signal transduction involving the activation of second messengers, such as cAMP, Ca^{2+} and diacylglycerol, thereby changing the mRNA expression[71]. Studies with glycosphingolipid have revealed the importance of these lipids and their metabolites in signaling pathways via the tyrosine kinase-linked receptors, a signal system mediated by protein kinase C (PKC), mitogen activated protein kinase (MAPK), other kinases, as well as mediated by the cytosolic Ca^{2+} concentration[72]. More recently, additional new signals involved in the adaptive intestinal response 3 days after a 50% intestinal resection have been identified by cDNA microarray analysis, such as small proline-rich protein 2, involved in wound healing; glutathione reductase, a gene involved in intestinal apoptosis; NF-2 family members, also involved in apoptosis; etoposide-induced p53-mediated apoptosis; basic Kruppe-like factor, a transcription factor that activates the promoter for IGF-1; and prothymosin-α, involved in cell proliferation[73,74]. These observations of altered expression of signals are useful to generate hypotheses that can be tested in future studies to establish whether these signals represent a primary or a secondary event.

The glycospingolipid, phospholipid, cholesterol and fatty acid composition of plasma membranes may be modified in mammalian cells[75]. For example, Keelan et al. (1990) demonstrated that alterations in dietary fatty acid saturation influence intestinal BBM phospholipid fatty acid composition in rats[76]. The investigators proposed that the previously reported diet-associated changes in active and passive intestinal transport are due at least in part to these alterations in the fatty acid composition in BBM phospho-

lipids. A diet enriched with SFA is associated with increases in the saturation of BBM phospholipid fatty acids, while a diet enriched with PUFA is associated with an increase in the unsaturation of BBM phospholipid fatty acids[3,4]. The degree of fatty acid unsaturation or saturation, as well as the cholesterol and ganglioside/glycosphingolipid content, are factors that influence the fluidity of the BBM[77,78]. Changes in the fluidity of the BBM may alter the permeation of molecules and nutrients through this barrier, as well as the conformation of binding sites on transporter proteins such as SGLT1 and GLUT5[79]. For example, alterations in BBM fluidity influence the passive uptake of lipids, as well as the carrier-mediated D-glucose uptake[79,80]. While enhancement of fluidity increases the uptake of lipids, fluidization of BBM from enterocytes located on the villous tip decreases the uptake of D-glucose to levels seen in the BBM from enterocytes located on the crypts[81]. The explanation for the effect of BBM fluidity on glucose uptake is unknown, but represents a potentially important post-translational process.

The lipid composition of cell membranes alters the passive permeability properties and transporter activity across the membrane[75]. The altered membrane lipid composition may act in part by changing the viscosity or fluidity of the membrane, including the microenvironment surrounding the transporter. Meddings (1989) compared *in vivo* membrane lipid permeability within the same intestinal region, under conditions where membrane physical properties were radically altered by feeding rats an inhibitor of cholesterol synthesis[82]. Marked reductions in membrane fluidity were observed due to the replacement of membrane cholesterol with its precursor 7-dehydrocholesterol. Associated with these alterations was a pronounced reduction in membrane lipid permeability. Therefore, BBM membrane lipid permeability*, in vivo*, appears to be correlated with the physical properties of the bilayer.

Recently, two types of specialized microdomains in the BBM have been identified: lipid rafts and caveolae. These regions are important in signal transduction as well as lipid and protein trafficking[83-85]. They are enriched in saturated fatty acids, cholesterol and gangliosides[84-86]. Feeding rats a diet enriched with gangliosides increases jejunal glucose uptake[87]. Feeding a ganglioside-rich diet increases the ganglioside content and decreases the cholesterol content in the intestinal mucosa, plasma, retina and brain[88]. Similar changes in the lipid composition of intestinal microdomains, or lipid rafts, occur following ganglioside feeding[89]. Although SGLT1 has been localized to these microdomains in renal epithelial cells[90], it is not known if sugar transporters reside in intestinal BBM microdomains. If this is the case, local changes in membrane fatty acids may affect the activity of transporter by altering the configuration of the protein, potentially exposing or masking the transporter binding sites and thereby modifying nutrient uptake. In addition, gangliosides may influence intestinal sugar transport *via* their effect on pro-inflammatory mediators, many of which are known to influence intestinal sugar transport[91-93]. For example, in rats challenged with lipopolysaccharide, ganglioside feeding reduced the production of intestinal platelet activating factor, PGE2, LTB4, as well as reduced plasma levels of IL-1β and TNF-α[94].

Carbohydrates

Dietary carbohydrate may induce the intestinal adaptive response by increasing the abundance of hexose transporters to facilitate a higher rate of sugar absorption[11]. In a murine model, intestinal glucose uptake was directly correlated with the dietary carbohydrate load[31,39,95]. The effect of dietary carbohydrate on nutrient transporter abundance has been reported in several animal models. For instance, the abundance of SGLT-1 in BBM and GLUT2 in the BLM were elevated in animals fed a high carbohydrate diet and associated with this enhanced level of protein was an increase in glucose absorption[19,96,97]. As well, the GLUT5 transporter abundance was elevated with enhanced dietary fructose, leading to increased fructose uptake[98].

The initiation of the dietary glucose-induced adaptive response occurs in the intestinal crypts, where the transport capacities of the nutrient transporters are programmed[39,40,95,97]. In this mouse model, phlorizin binding was utilized as a means of measuring the glucose transporter site density. Changing the murine diet from a high to a low carbohydrate regimen reduced the amount of glucose transporter, as estimated from the density of phlorizin binding. The alteration in the density of phlorizin binding was first observed in the crypt cells, and over a three-day period was subsequently seen in the villous tip cells. This suggests that the crypt enterocytes respond to the high carbohydrate diet to increase their phloridzin binding; those cells then migrate up the villous over the next three days, contributing to the process of enhancing glucose uptake.

The enterocytes may adapt to the high carbohydrate diet by increasing the crypt cell turnover rate, enhancing the enterocyte migration rate, as well as by reprogramming the capability of nutrient transporters in the crypts to accommodate to the requirement for higher monosaccharide transporters[97].

Animals fed a glucose-enriched diet have an increased glucose uptake, resulting from up-regulation of both BBM and BLM glucose transporters[19,96,99]. The precocious introduction of dietary fructose causes enhanced expression of fructose transporters and fructose transport earlier during development, without changing glucose uptake[97]. The substrates glucose and fructose are both specific in terms of up-regulation of their corresponding transporters, SGLT1 and GLUT5. Therefore, increasing the sugar composition of the diet results in increases in the transport of these nutrients. In contrast, increases in essential amino acids or other substances that are potentially toxic at high levels (such as iron, calcium or phosphorous) are associated with no change, or even reductions in transport[38,100].

Furthermore, in many cases other nutrients may be equal or even more potent inducers of the transporter than its specific substrate. For example, young animals fed a diet enriched with polyunsaturated fatty acids (PUFA) have a decline in glucose uptake, as compared to animals fed a saturated fatty acid (SFA) enriched diet[70,101,102]. Similarly, Vine *et al* (2002) studied the effect of various fatty acids on the passive and active transport properties of rat jejunum, and found that an SFA-enriched diet increased Na^+-dependent glucose uptake when compared to a diet

enriched with n6 PUFA[103]. In contrast, in aged rats, glucose uptake is increased by PUFA and not by SFA[36].

Dietary fiber also modulates intestinal nutrient uptake. For example, a diet enriched with fermentable fiber increased glucose uptake and GLUT2 transporter abundance in dogs[104]. *In vitro* studies, in which rat intestinal tissue was incubated with β-glucan isolated from barley or oats, show reductions in the uptake of stearic and linoleic acids (Drozdowski *et al*, 2005, unpublished observations). Furthermore, many studies have investigated the effect of TPN supplemented with short chain fatty acids, the products of fiber fermentation. Increases in glucose uptake, GLUT2 mRNA and protein, and intestinal morphology were seen in normal rats as well as in rats following intestinal resection[105-108].

Protein

Dietary protein also has an impact on the intestinal morphology and active amino acid transport[40,109,110]. Both *in vitro*[110] and *in vivo*[109] rat experiments have shown that a high protein diet increases amino acid uptake in the jejunum. An alteration in the amount of dietary protein induces reversible adaptation of the non-essential amino acid transport rate[111]. Feeding a high protein diet to mice induces a 77%-81% increment in the uptake of non-essential amino acids[40], yet only a 32%-61% increase for essential amino acids. On the other hand, a protein-deficient regimen reduces uptake of non-essential amino acids, such as aspartate and proline, and maintains or increases uptake for essential amino acids and alanine. Thus, the nature of the adaptive response depends upon the type of amino acid and the needs of the animal.

Glutamine is a key metabolic fuel for enterocytes, mediating cellular nucleic acid synthesis and proliferation. Parenterally fed rats demonstrate decreased atrophy of the intestinal mucosa following glutamine supplementation[112]. Glutamine administration also normalizes the reduced levels of intestinal adaptation in rats receiving total parenteral nutrition (TPN) following intestinal resection[113]. It is noteworthy that some studies of oral glutamine supplementation in the rat have failed to document more than temporary mucosal proliferation[114]. This indicates that mechanistic differences that are intrinsic to the method of glutamine administration may exist, and suggests that these may be significant in regulating the adaptive response.

Other amino acids may inhibit intestinal adaptation. Sukhotnik *et al* (2005) examined the effects of the parenteral administration of the nitric oxide precursor arginine to rats following 75% small bowel resection[115]. Arginine supplementation was associated with lower cell proliferation indexes and greater enterocyte apoptosis. This observation led the investigators to conclude that arginine inhibits structural intestinal adaptation.

Polyamines

Polyamines are found in all eukaryotic cells[116], and they play an important role in growth and differentiation[117]. Polyamines are obtained either from the diet, or via synthesis from ornithine[118]. Uda *et al* (2002) demonstrated that luminal perfusions of polyamines rapidly (in less than 5 min) enhance intestinal glucose uptake in rats, and increase BBM SGLT1 protein[119].

Polyamine synthesis or uptake may be an important event that initiates the adaptive hyperplasia seen in the intestinal remnant after partial small bowel resection. Enteral diets supplemented with ornithine alpha-ketoglutarate (OKG), a precursor for arginine, glutamine and polyamines, enhances intestinal adaptation in models of intestinal resection[120,121]. Indeed, studies by both Tappenden *et al*[122] and Thiesen *et al*[66] suggest that ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis, may mediate the adaptive process in rats that is stimulated by the administration of either glucocorticosteroids or short chain fatty acids to rats following intestinal resection.

The role of polyamines in the adaptation of the intestine during development has also been studied. Wild *et al* (2005, unpublished observations) showed that in postnatal rats, oral spermidine treatment resulted in the precocious expression of the intestinal sugar transporters (SGLT1, GLUT2 and GLUT5), as well as ODC. This led the investigators to conclude that oral polyamines induce precocious maturation of sugar transporters, which may be mediated by alterations in ODC gene expression.

HORMONAL REGULATION

Glucocorticosteroids

In a model of extensive intestinal resection (50% enterectomy), the proximal and distal intestinal remnants were adequate to assess the morphology and function at these sites[11,112]. The glucocorticosteroid prednisone had no effect on the intestinal uptake of glucose or fructose in these resected animals[70]. In contrast, the locally acting steroid budesonide increased by over 120% the value of the jejunal Vmax for the uptake of glucose, and increased by over 150% the ileal uptake of fructose. The protein abundance and mRNA expression of SGLT1, GLUT5, GLUT2 and Na+/K+ APTase α 1 and β 1 cannot explain the enhancing effect of budesonide on glucose and fructose uptake. Budesonide, prednisone and dexamethasone reduced the jejunal expression of the early response gene c-jun. In resected animals, the abundance of the mRNA of ODC in the jejunum was reduced, and glucocorticosteroids (GC) reduced the jejunal expression of the mRNA of proglucagon. These data suggest that the enhancing influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC and proglucagon, or other as yet unknown signals.

In contrast, the uptake of D-fructose by GLUT5 was similarly increased with budesonide and with prednisone. The increases in the uptake of fructose were not due to variations in the weight of the intestinal mucosa, food intake, or in GLUT5 protein or mRNA expression. There were no steroid-associated changes in mRNA expression of c-myc, c-jun, c-fos, of proglucagon, or of selected cytokines. However, the abundance of ileal ODC mRNA was increased with prednisone. Giving budesonide or prednisone to post- weaning rats for four weeks in doses equivalent to those used in clinical practice increases

fructose but not glucose uptake. This enhanced uptake of fructose was likely regulated by post-translational processes[70].

Growth hormone

Growth hormone (GH) has been suggested as possessing pro-adaptive properties[123]. In rats and piglets, GH administration results in an increase in small bowel length and function per unit length[124]. Hypophysectomized rats undergo mucosal hypoplasia of the small bowel, as well as a reduced adaptive response following resection that is restored by GH[125]. In contrast, transgenic mice expressing elevated levels of GH show hypertrophy of the small intestine[124]. IGF-1 expression in the small bowel is regulated by GH and is believed to induce enterotrophic effects following resection[113,126]. In a rat model of SBS, acute IGF-1 treatment of TPN fed rats produced sustained jejunal hyperplasia, and facilitated weaning from parenteral to enteral nutrition[127]. GH administration to normal rats has been reported to have positive effects on mucosal growth and intestinal adaptation following massive resection[128], although contradictory data exists[129,130]. Human and rabbit studies have indicated that increased nutrient transport activity devoid of morphologic changes may be the method of GH-induced intestinal adaptation[131].

GH administration has been shown to inhibit the liberation of glutamine from muscle during catabolic states in humans[132], suggesting a possible role for combined GH and glutamine provision in adaptive bowel enhancement. Trials investigating any such synergism in the rat have yielded conflicting results. Some studies have failed to demonstrate an additive effect of GH and glutamine in the enhancement of post-resection intestinal adaptation[133], while others have documented a positive synergistic effect[134].

The mechanisms of action by which GH and/or glutamine may enhance the human adaptive process cannot be clearly surmised from the existing rodent data. Many studies have inherent deficits in terms of nutrient controls that could have contributed to the conflicting outcomes which have been described. The trophic effects of enteral nutrition on the adaptation process are well known[3]. Studies evaluating the contribution of non-specific, nutrient-derived augmentation of the adaptation process, as well as the mechanisms of any such nutrient factor interactions, may be useful in defining more accurate and therapeutically applicable results.

Animal studies have confirmed the enhancing effect of GH on nutrient absorption[123,124]. For example, GH has been shown to enhance the absorption of amino acids using *ex vivo* human BBM vesicles[134]. An intestinal mucosal GH receptor has been described in rats and humans[135], and GH promotes cell differentiation and clonal expansion of these differentiated cells[136].

The use of growth hormone in human clinical studies has been investigated. It has been suggested that the efficacy of GH and/or glutamine therapy in the adaptive response of the small bowel may be based mainly upon the clinical status of the patient (for example, the presence of a portion of the colon in continuity with the remaining resected small intestine)[137]. Evaluation of the effect of such variables in the rat may facilitate further understanding of the pathology and physiology of the bowel adaptation process, as well as more clearly defining positive predictive indicators of the bowel's ability to be rehabilitated. Furthermore, existing human data has indicated that the administration of high concentrations of GH can actually increase patient morbidity and mortality[138], demonstrating a primary need for equivalent clinical research in the testing of these factors.

In home parenteral nutrition (HPN)-dependent patients with SBS, the use of high dose recombinant human GH (0.4 mg/kg per day) in controlled[138,139] and uncontrolled studies[140] has led to variable results. These patients were given glutamine supplements by mouth or parenterally, and their diet was modified. In the randomized and placebo-controlled study of Scolapio *et al*[139], the subjects ingested a standardized 1500 kcal/d diet, which is clearly different from the hyperphagic diet consumed by many SBS patients[140], and which may contribute to the physiological adaptation that occurs in the remaining intestine after extensive resection. It is unclear whether glutamine is beneficial for the adaptive response in humans, and in rat models of SBS, it is unclear whether glutamine supplementation is efficacious for the adaptive process[2,141]. Furthermore, both a hyperphagic diet and the absence of malnutrition are needed for humans to achieve optimal intestinal adaptation[44,142].

When HPN-dependent patients with SBS were provided a usual *ad libitum* hyperphagic diet, and given low doses of GH (0.05 mg/kg per day) for three weeks, there was significant improvement in the intestinal absorption of energy (15% ± 5%), nitrogen (14% ± 6%) and carbohydrate (10% ± 4%)[143]. The increased food absorption represented 37% ± 16% of total parenteral energy delivery. Body weight, lean body mass, D-xylose absorption, insulin-like growth factor 1, and insulin-like growth factor binding protein 3 increased, whereas uptake of GH binding protein decreased. During treatment with GH, improvement in net intestinal absorption compared with placebo was 427 ± 87 kcal/d, representing 19% ± 8% of the total energy expenditure required to obtain energy balance equilibrium in patients with SBS[140].

A review of the literature in this area by Matarese *et al*[144] noted that there were differences in gastrointestinal (GI) anatomy, dietary compliance, nutritional status, presence of mucosal disease, and diagnosis both within and between the studies. They concluded that "*administering recombinant human growth hormone alone or together with glutamine with or without a modified diet may be of benefit when the appropriate patients are selected for treatment*".

Insulin-like growth factor 1

Insulin-like growth factor 1 (IGF-1) also proved to be efficient in increasing intestinal adaptation following resection in rats. IGF-1 treatment following 70% jejuno-ileal resection attenuated fat and amino acid malabsorption[145] and increased total gut weight by up to 21%. The IGF-1 receptor was increased in the jejunum and

colon due to resection. Resection also increased circulating IGF-binding proteins (IGFBP). IGF-1 treatment had no effect on IGF-1 mRNA or IGF-1 receptor density, but increased IGFBP5 in the jejunum. This increase in IGFBP5 was correlated with jejunal growth after IGF-1 treatment[146].

More recently, a study was conducted to determine the effect of IGF-1 on enterocyte kinetics following intestinal resection[147]. IGF-I treatment in resected rats significantly increased jejunal mucosal mass by 20% and mucosal concentrations of protein and DNA by 36% and 33%, respectively, above the response to resection alone. These changes reflected an increase in enterocyte proliferation and an expansion of the proliferative compartment in the crypt. No further decrease in enterocyte apoptosis and increase in enterocyte migration were observed.

IGF-I treatment may also facilitate weaning from parenteral to enteral nutrition. After a 60% jejunoileal resection plus cecectomy, rats treated with recombinant human IGF-I (3 mg/kg body weight) or control vehicle were maintained exclusively with TPN for 4 d and were then transitioned to oral feeding. TPN and IGF-I were stopped 7 d after resection and rats were maintained with oral feeding for 10 more days. Acute IGF-I treatment induced sustained jejunal hyperplasia, as demonstrated from the presence of greater concentrations of both jejunal mucosal protein and DNA, and was associated with the maintenance of a greater body weight and serum IGF-I concentrations[127].

A study was done using male transgenic mice with targeted smooth muscle IGF-1 overexpression[148]. These animals and non-transgenic littermates underwent 50% proximal small bowel resection. The results showed that growth factor over-expression led to a unique mucosal response characterized by a persistent increase in remnant intestinal length and an increase in mucosal surface area. Therefore, IGF-1 signaling from within the muscle layer may be important in resection-induced intestinal adaptation.

Epidermal growth factor

Epidermal growth factor (EGF) up-regulates intestinal nutrient transport[149]. This effect is mediated by PKC and PI3K[150] and involves the redistribution of SGLT1 from microsomal pools to the BBM[151]. After massive intestinal resection, endogenous EGF is increased in the saliva and is decreased in the urine[152]. EGF stimulates intestinal adaptation after intestinal resection: the BBM surface area and the total absorptive area increased until d 10, and EGF treatment induced a further increase in BBM surface area[153]. In a study by O'Brien and colleagues[154], mice underwent a 50% small bowel resection or sham operation, and were then given orally an epidermal growth factor receptor (EGFR) inhibitor (ZD1839, 50 mg/kg per day) or control vehicle for 3 d. ZD1839 prevented EGFR activation, as well as the normal postresection increases in ileal wet weight, villus height, and crypt depth. Enterocyte proliferation was reduced two-fold in the resection group by ZD1839. These results more directly confirm the requirement of a functional EGFR as a mediator of the postresection adaptation response. Interestingly, previous work has demonstrated that the EGFR is predominantly located on the BLM of enterocytes[155], but after small bowel resection the EGFR shows redistribution from the BLM to the BBM, with no change in the total amount of EGFR[156]. It is not known how this redistribution occurs. This is an important point, since modification of this process may represent a useful means to accelerate the intestinal adaptive process.

In a study by Knott *et al*[157], laser capture microdissection (LCM) microscopy was used to elucidate the specific cellular compartment(s) responsible for postresection changes in EGFR expression. Mice underwent a 50% proximal resection or sham operation, and after three days frozen sections were taken from the remnant ileum. Individual cells from the villi, crypt, muscularis and mesenchymal compartments were isolated. EGFR mRNA expression for each cell compartment was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR). EGFR expression was increased two-fold in the crypt after resection, directly correlating with the zone of cell proliferation. This supports the hypothesis that EGFR signaling is crucial for the mitogenic stimulus for adaptation. The additional finding of increased EGFR expression in the muscular compartment is novel, and may imply a role for EGFR in the muscular hyperplasia seen after massive small bowel resection. As noted previously, it is of interest that the muscle layer also appears to play a role in the adaptive response to IGF-1[148].

The treatment of resected rats with EGF has been studied. In a study by Sham *et al*[158], male juvenile rats underwent either transection or ileocecal resection leaving a 20-cm jejunal remnant. Resected animals were treated orally with placebo or recombinant human EGF. Resected EGF-treated animals lost significantly less weight than those in the transection group, absorbed significantly more 3-0 methylglucose, and had reduced intestinal permeability as determined by the lactulose/mannitol ratio. Work by Chung *et al*[159] using rabbits showed that intestinal resection altered SGLT1 mRNA and protein expression along the crypt-villous axis, with expression being highest in the mid-villous region. Oral EGF normalized SGLT1 expression, resulting in a gradient of increasing expression from the base of the villus to the villous tip.

More recently, Nakai and colleagues[160] investigated the role of EGF in stimulating intestinal adaptation following small bowel transplantation. Treatment of rats with EGF (intraperitoneally for three days) following intestinal transplantation resulted in increased glucose absorption, SGLT1 abundance and the villous height and crypt depth in the graft. This has not yet been studied in humans.

Keratinocyte growth factor

In a study by Yang *et al*[161], adult C57BL/6J mice were randomized to a 55% mid-small bowel resection, resection with keratinocyte growth factor (KGF) administration (SBSKGF), or a sham-operated (control) group, and were killed at d 7. Ussing chamber studies showed that KGF increased the net transepithelial absorption of 3-O-methyl glucose as well as sodium-coupled alanine absorption, but

had no effect on epithelial permeability barrier function. Epithelial cells were separated along the crypt-villous axis with LCM, and epithelial KGF receptor (KGFR) mRNA abundance was studied using real time RT-PCR. KGF up-regulated KGFR mRNA abundance, predominately in the crypt and the lower portion of the villus.

Leptin

Leptin plays an important role in the regulation of body fat and satiety (reviewed in[162]). Leptin reduces food intake[163] and leptin-deficient mice develop obesity[164]. Leptin may be a potential growth factor for the normal rat small intestine. The effect of 14 d of parenteral leptin administration (2 μg/kg per day) to rats following 80% small bowel resection was studied. Leptin was associated with a 44% increase in galactose absorption and a 14% increase in GLUT-5 abundance, but with no change in DNA content or in SGLT abundance. These findings suggest that leptin may potentially be clinically useful in patients with impaired intestinal function[165].

Ghrelin

Ghrelin is a gastric hormone that is released in response to enteral nutrients. It has an opposite effect when compared to leptin, as it stimulates food intake[166]. The role of ghrelin in intestinal adaptation is unknown.

Glucagon-like peptide 2

Animal studies have demonstrated a potential role for GLP-2 in the adaptive response following intestinal resection[147]. Several investigators have demonstrated increases in plasma GLP-2 levels following intestinal resection in rats[167-169]. In a study by Dahly *et al*[147], rats were subjected to 70% midjejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 d post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats[147].

Recently, further correlations between post-resection GLP-2 levels, morphological indices, crypt cell proliferation rates and nutrient absorption have been made[170]. In this study, an inverse correlation was found between post-prandial GLP-2 levels and fat or protein absorption as assessed by a 48 h balance study. These results, along with data obtained from studies showing that GLP-2 immunoneutralization inhibits post-resection adaptation[171], further implicate GLP-2 as a post-resection mediator of adaptation.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection[172]. In this study, Sprague-Dawley rats were divided into two groups, with a 75% mid-jejunum-ileum resection and a sham operated groups. Animals were treated with 0.1 μg/g GLP-2 analog (protease resistant human GLP-2) or placebo given subcutaneously twice daily for 21 d. The total weight of the rats and the mucosal mass per centimeter were compared between the groups. Administration of this peptide or its analogs was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum. The absorption and urinary excretion of oral D-xylose is proportional to intestinal mucosal surface area and transit time. While resection reduced D-xylose excretion and GLP-2 restored D-xylose excretion to levels above control values within 21 d of administration. This indicates that GLP-2 has a positive effect on intestinal morphology and absorptive function following resection.

More recently, Martin *et al*[173] investigated the effects of GLP-2 in a TPN-supported model of experimental short bowel syndrome. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, and TPN plus 10 μg/kg per hour^{GLP-2}. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villus height, intestinal mucosal surface area, and crypt cell proliferation. Intestinal permeability tests showed that GLP-2 reduced the lactulose-mannitol ratio indicating that GLP-2 lowered intestinal permeability when compared with the TPN alone. GLP-2 increased serum GLP-2 levels and intestinal SGLT-1 protein abundance compared with either TPN or enteral groups. This study demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding,

Because a number of hormones and growth factors have been shown to influence intestinal function, Washizawa *et al*[174] compared the effects of GLP-2, GH and KGF on markers of gut adaptation following massive small bowel resection (MSBR). KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in the small bowel and the colon. They also observed that while both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following MSBR, the authors conclude that a combination of these agents may be most beneficial.

A pilot study to determine the efficacy of GLP-2 in patients with SBS has been completed. A non-placebo controlled study was conducted in 8 patients with SBS with an end-enterostomy type of anastomasis (6 had Crohn's disease and 4 were not receiving HPN)[175]. Treatment with GLP-2 (400 μg subcutaneously twice a day for 35 d) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in 5 and 6 patients, respectively.

A review by Jeppesen[176] on the role of GLP-2 in the treatment of SBS concludes that *"Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research studies. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of*

parenteral nutrition in a larger fraction of short-bowel patients".

SIGNALS OF INTESTINAL ADAPTATION

A number of studies have investigated the signals involved in intestinal adaptation using animal models of intestinal resection. Dodson *et al*[177] identified three subsets of genes that were up-regulated by constructing a cDNA library from the remnant ileum of resected rats. This library was screened, and subtractive hybridization was used to identify genes that were induced following resection. These included genes involved with regulating the absorption and metabolism of nutrients. For example, L-FABP, apolipoprotein A-IV, cellular retinal binding protein II and ileal lipid binding protein were identified as genes that were induced following 70% intestinal resection in rats. Genes involved in cell cycle regulation were also identified. For example, phosphorylation and dephosphorylation are important regulators of the cell cycle, and PP1δ, a subunit of a serine/threonine phosphatase was indeed up-regulated. Grp78, a member of the heat shock protein family was also increased. Grp78 resides in the ER and acts as a chaperone during protein assembly and transport. It may also have a protective role, and prevent apoptosis as a way of promoting the proliferative response following intestinal resection[178,179].

Rubin *et al*[180] further characterized the molecular and cellular mechanisms following 70% resection in rats. An immediate early gene, PC4/TIS7, was markedly increased soon after resection, with levels returning back to normal by one week post-resection. Although the function of this protein is unknown, it may be related to cytodifferentiation as it is expressed only in the villus and not in the crypts.

Erwin *et al*[74] used cDNA microarrays to gain insight into the mechanism of intestinal adaptation. Mice underwent a 50% intestinal resection and three days afterwards RNA was extracted from the remnant ileum. Multiple genes were induced, and they divided these into four categories: (1) Apoptosis, DNA synthesis, repair and recombination (10 genes); (2) Oncogenes, tumor suppressors, cell cycle regulators (3 genes); (3) Stress response, ion channels and transport (4 genes); (4) Transcription factors and general DNA-binding proteins (1 gene). Many of the genes (ODC, c-neu, glucose-related protein, IGFBP-4) that were identified agreed with the results of other studies of intestinal resection. For example, ODC was increased in this study, and this agrees with previous findings that showed ODC to be involved in the adaptive process[66,122,181]. Some new factors were also identified including glutathione reductase (involved in apoptosis), Basic Kruppel-like factor (transcriptional regulator that activates the IGF promoter), prothymosin-α (associated with increased cell proliferation), and eteposide induced p53 responsive mRNA (stress response protein involved in p53 mediated apoptosis).

Stern *et al*[73] performed a similar analysis of gene expression following 50% intestinal resection in rats. The gene with the largest increase was identified as *sprr2*, a novel gene unknown previously to be involved in intestinal adaptation. EGF administration post-resection further increased *sprr2* expression and enhanced the adaptive response. This protein plays a role in the terminal differentiation of stratified squamous epithelium. Its role in the intestinal epithelium is unclear and warrants further investigation.

Finally, a variety of other signals have been described as possibly playing a role in the process of intestinal adaptation. These include prostanoids[182], uncoupling proteins[183], peroxisome proliferation-activated receptor α (PPARα)[184], transforming growth factor-α[185], SPARC (secreted protein, acidic and rich in cystine[186], Bcl-2[187], endothelin-1[188], erythropoietin[189], the GATA family of zinc finger transcription factors[190], hepatocyte growth factor[191], the early response genes (ERG)[192], PC4/TIS7[180] and epimorphin[193]. More recently, augmented Wnt signaling has been shown to enhance the adaptive response to massive small bowel resection[194]. Several of these signals may be useful to modify in a clinical setting to enhance the intestinal adaptive response.

Microarray technology is a powerful tool that is constantly developing into a more sophisticated technique of identifying novel genes involved in physiological processes. Intestinal adaptation awaits further characterization by hypothesis-testing studies. From the information that is available at this time, it is clear that genes regulating the cell cycle, proliferation, differentiation and apoptosis are important components of the adaptive process.

CONCLUSION

The process of intestinal adaptation is complex and multifaceted. Although a number of trophic nutrients and non-nutritive factors have been identified in animal studies, successful and reproducible clinical trials are lacking. However, the functional adaptation of segmental bowel grafts has been studied. Benedetti *et al* (2001) reported a case study in which a segmental graft from a living donor demonstrated preogressive functional adaptation, as assessed by water and D-xylose absorption as well as fecal fat studies[195]. Morphological adaptation has been documented in three patients following successful intestinal transplantation[196]. In fact, more than 50% increases in villous height and absorptive surface area were seen at 6 mo, suggesting that functional changes in intestinal function are associated with morphological changes. Therefore, while human data is limited there is evidence indicating that the adapative process occurs. Still, additional human studies are required before the clinical implications of the animal data can truly be established. Understanding the mechanisms underlying the adaptive process in humans may direct research toward strategies that maximize intestinal function and impart a true clinical benefit to patients with short bowel syndrome.

REFERENCES

1 **Nightingale J**. Intestinal Failure. London: Greenwich Medical Media Limited, 2001

2 **Vanderhoof JA**, Langnas AN. Short-bowel syndrome in children and adults. *Gastroenterology* 1997; **113**: 1767-1778

3 **Thomson AB**, Wild G. Adaptation of intestinal nutrient transport in health and disease. Part I. *Dig Dis Sci* 1997; **42**:

453-469
4 **Thomson AB**, Wild G. Adaptation of intestinal nutrient transport in health and disease. Part II. *Dig Dis Sci* 1997; **42**: 470-488
5 **Ferraris RP**, Carey HV. Intestinal transport during fasting and malnutrition. *Annu Rev Nutr* 2000; **20**: 195-219
6 **Thiesen A**, Drozdowski L, Iordache C, Neo CC, Woudstra TD, Xenodemetropoulos T, Keelan M, Clandinin MT, Thomson AB, Wild G. Adaptation following intestinal resection: mechanisms and signals. *Best Pract Res Clin Gastroenterol* 2003; **17**: 981-995
7 **Sturm A**, Layer P, Goebell H, Dignass AU. Short-bowel syndrome: an update on the therapeutic approach. *Scand J Gastroenterol* 1997; **32**: 289-296
8 **Burant CF**, Flink S, DePaoli AM, Chen J, Lee WS, Hediger MA, Buse JB, Chang EB. Small intestine hexose transport in experimental diabetes. Increased transporter mRNA and protein expression in enterocytes. *J Clin Invest* 1994; **93**: 578-585
9 **Wolvekamp MC**, Heineman E, Taylor RG, Fuller PJ. Towards understanding the process of intestinal adaptation. *Dig Dis* 1996; **14**: 59-72
10 **O'Connor TP**, Lam MM, Diamond J. Magnitude of functional adaptation after intestinal resection. *Am J Physiol* 1999; **276**: R1265-R1275
11 **Diamond JM**, Karasov WH, Cary C, Enders D, Yung R. Effect of dietary carbohydrate on monosaccharide uptake by mouse small intestine in vitro. *J Physiol* 1984; **349**: 419-440
12 **Ferraris RP**, Diamond JM. Specific regulation of intestinal nutrient transporters by their dietary substrates. *Annu Rev Physiol* 1989; **51**: 125-141
13 **Ferraris RP**, Diamond J. Regulation of intestinal sugar transport. *Physiol Rev* 1997; **77**: 257-302
14 **Kellett GL**, Helliwell PA. The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *Biochem J* 2000; **350** Pt 1: 155-162
15 **Helliwell PA**, Richardson M, Affleck J, Kellett GL. Regulation of GLUT5, GLUT2 and intestinal brush-border fructose absorption by the extracellular signal-regulated kinase, p38 mitogen-activated kinase and phosphatidylinositol 3-kinase intracellular signalling pathways: implications for adaptation to diabetes. *Biochem J* 2000; **350** Pt 1: 163-169
16 **Thomson AB**, McIntyre Y, MacLeod J, Keelan M. Dietary fat content influences uptake of hexoses and lipids into rabbit jejunum following ileal resection. *Digestion* 1986; **35**: 78-88
17 **Keelan M**, Walker K, Thomson AB. Resection of rabbit ileum: effect on brush border membrane enzyme markers and lipids. *Can J Physiol Pharmacol* 1985; **63**: 1528-1532
18 **Wild GE**, Thompson JA, Searles L, Turner R, Hasan J, Thomson AB. Small intestinal Na+,K+-adenosine triphosphatase activity and gene expression in experimental diabetes mellitus. *Dig Dis Sci* 1999; **44**: 407-414
19 **Cheeseman CI**, Maenz DD. Rapid regulation of D-glucose transport in basolateral membrane of rat jejunum. *Am J Physiol* 1989; **256**: G878-G883
20 **Tsang R**, Ao Z, Cheeseman C. Influence of vascular and luminal hexoses on rat intestinal basolateral glucose transport. *Can J Physiol Pharmacol* 1994; **72**: 317-326
21 **Maenz DD**, Cheeseman CI. Effect of hyperglycemia on D-glucose transport across the brush-border and basolateral membrane of rat small intestine. *Biochim Biophys Acta* 1986; **860**: 277-285
22 **Corpe CP**, Basaleh MM, Affleck J, Gould G, Jess TJ, Kellett GL. The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes. *Pflugers Arch* 1996; **432**: 192-201
23 **Dowling RH**, Booth CC. Structural and functional changes following small intestinal resection in the rat. *Clin Sci* 1967; **32**: 139-149
24 **Rand EB**, Depaoli AM, Davidson NO, Bell GI, Burant CF. Sequence, tissue distribution, and functional characterization of the rat fructose transporter GLUT5. *Am J Physiol* 1993; **264**: G1169-G1176
25 **Thomson AB**, Cheeseman CI, Keelan M, Fedorak R, Clandinin MT. Crypt cell production rate, enterocyte turnover time and appearance of transport along the jejunal villus of the rat. *Biochim Biophys Acta* 1994; **1191**: 197-204
26 **Park JH**, McCusker RH, Mohammadpour H, Blackwood DJ, Hrbek M, Vanderhoof JA. Dexamethasone inhibits mucosal adaptation after small bowel resection. *Am J Physiol* 1994; **266**: G497-G503
27 **Smith MW**. Autoradiographic analysis of alanine uptake by newborn pig intestine. *Experientia* 1981; **37**: 868-870
28 **Fedorak RN**, Cheeseman CI, Thomson AB, Porter VM. Altered glucose carrier expression: mechanism of intestinal adaptation during streptozocin-induced diabetes in rats. *Am J Physiol* 1991; **261**: G585-G591
29 **Musacchia XJ**, Hartner AM. Intestinal absorption of glucose, and blood glucose and hematocrit in pregnant and nonpregnant hamsters. *Proc Soc Exp Biol Med* 1970; **135**: 307-310
30 **Cripps AW**, Williams VJ. The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. *Br J Nutr* 1975; **33**: 17-32
31 **Sanderson IR**, Naik S. Dietary regulation of intestinal gene expression. *Annu Rev Nutr* 2000; **20**: 311-338
32 **Fischer E**, Lauterbach F. Effect of hyperglycaemia on sugar transport in the isolated mucosa of guinea-pig small intestine. *J Physiol* 1984; **355**: 567-586
33 **Keelan M**, Walker K, Thomson AB. Intestinal brush border membrane marker enzymes, lipid composition and villus morphology: effect of fasting and diabetes mellitus in rats. *Comp Biochem Physiol A Comp Physiol* 1985; **82**: 83-89
34 **Dinda PK**, Beck IT. Ethanol-induced inhibition of glucose transport across the isolated brush-border membrane of hamster jejunum. *Dig Dis Sci* 1981; **26**: 23-32
35 **Robinson JW**, Van Melle G, Riecken EO, Menge H. Structural and functional correlations in the hypertrophic mucosa of intestinal remnants following resection in rats. *Res Exp Med* (Berl) 1982; **181**: 95-104
36 **Drozdowski L**, Woudstra T, Wild G, Clandindin MT, Thomson AB. The age-associated decline in the intestinal uptake of glucose is not accompanied by changes in the mRNA or protein abundance of SGLT1. *Mech Ageing Dev* 2003; **124**: 1035-1045
37 **Thomson AB**, Cheeseman CI, Walker K. Effect of abdominal irradiation on the kinetic parameters of intestinal uptake of glucose, galactose, leucine, and gly-leucine in the rat. *J Lab Clin Med* 1983; **102**: 813-827
38 **Diamond JM**, Karasov WH. Adaptive regulation of intestinal nutrient transporters. *Proc Natl Acad Sci USA* 1987; **84**: 2242-2245
39 **Karasov WH**, Diamond JM. Adaptive regulation of sugar and amino acid transport by vertebrate intestine. *Am J Physiol* 1983; **245**: G443-G462
40 **Karasov WH**, Solberg DH, Diamond JM. Dependence of intestinal amino acid uptake on dietary protein or amino acid levels. *Am J Physiol* 1987; **252**: G614-G625
41 **Pullan JM**. Massive intestinal resection. *Proc R Soc Med* 1959; **52**: 31-37
42 **Weinstein LD**, Shoemaker CP, Hersh T, Wright HK. Enhanced intestinal absorption after small bowel resection in man. *Arch Surg* 1969; **99**: 560-562
43 **Howard L**, Hassan N. Home parenteral nutrition. 25 years later. *Gastroenterol Clin North Am* 1998; **27**: 481-512
44 **Thompson JS**, Langnas AN, Pinch LW, Kaufman S, Quigley EM, Vanderhoof JA. Surgical approach to short-bowel syndrome. Experience in a population of 160 patients. *Ann Surg* 1995; **222**: 600-605; discussion 600-605
45 **Ziegler TR**, Fernández-Estívariz C, Gu LH, Bazargan N, Umeakunne K, Wallace TM, Diaz EE, Rosado KE, Pascal RR, Galloway JR, Wilcox JN, Leader LM. Distribution of the H+/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. *Am J Clin Nutr* 2002; **75**: 922-930
46 **Pironi L**, Paganelli GM, Miglioli M, Biasco G, Santucci R, Rug-

geri E, Di Febo G, Barbara L. Morphologic and cytoproliferative patterns of duodenal mucosa in two patients after long-term total parenteral nutrition: changes with oral refeeding and relation to intestinal resection. *JPEN J Parenter Enteral Nutr* 1994; **18**: 351-354

47 **Porus RL**. Epithelial hyperplasia following massive small bowel resection in man. *Gastroenterology* 1965; **48**: 753-757

48 **Kararli TT**. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos* 1995; **16**: 351-380

49 **Gleeson MH**, Cullen J, Dowling RH. Intestinal structure and function after small bowel by-pass in the rat. *Clin Sci* 1972; **43**: 731-742

50 **Benhamou PH**, Canarelli JP, Richard S, Cordonnier C, Postel JP, Grenier E, Leke A, Dupont C. Human recombinant growth hormone increases small bowel lengthening after massive small bowel resection in piglets. *J Pediatr Surg* 1997; **32**: 1332-1336

51 **Heemskerk VH**, van Heurn LW, Farla P, Buurman WA, Piersma F, ter Riet G, Heineman E. A successful short-bowel syndrome model in neonatal piglets. *J Pediatr Gastroenterol Nutr* 1999; **29**: 457-461

52 **Lauronen J**, Pakarinen MP, Kuusanmäki P, Savilahti E, Vento P, Paavonen T, Halttunen J. Intestinal adaptation after massive proximal small-bowel resection in the pig. *Scand J Gastroenterol* 1998; **33**: 152-158

53 **Heemskerk VH**, van Heurn LW, Farla P, Buurman WA, Piersma F, ter Riet G, Heineman E. Effect of IGF-rich colostrum on bowel adaptation in neonatal piglets with short bowel syndrome. *J Pediatr Gastroenterol Nutr* 2002; **34**: 47-51

54 **Bines JE**, Taylor RG, Justice F, Paris MC, Sourial M, Nagy E, Emselle S, Catto-Smith AG, Fuller PJ. Influence of diet complexity on intestinal adaptation following massive small bowel resection in a preclinical model. *J Gastroenterol Hepatol* 2002; **17**: 1170-1179

55 **Sanderson IR**. Dietary regulation of genes expressed in the developing intestinal epithelium. *Am J Clin Nutr* 1998; **68**: 999-1005

56 **Jump DB**, Clarke SD. Regulation of gene expression by dietary fat. *Annu Rev Nutr* 1999; **19**: 63-90

57 **Diamond JM**. Evolutionary physiology. Quantitative design of life. *Nature* 1993; **366**: 405-406

58 **Lam MM**, O'Connor TP, Diamond J. Loads, capacities and safety factors of maltase and the glucose transporter SGLT1 in mouse intestinal brush border. *J Physiol* 2002; **542**: 493-500

59 **Levine GM**, Deren JJ, Steiger E, Zinno R. Role of oral intake in maintenance of gut mass and disaccharide activity. *Gastroenterology* 1974; **67**: 975-982

60 **Johnson LR**, Copeland EM, Dudrick SJ, Lichtenberger LM, Castro GA. Structural and hormonal alterations in the gastrointestinal tract of parenterally fed rats. *Gastroenterology* 1975; **68**: 1177-1183

61 **Hosoda N**, Nishi M, Nakagawa M, Hiramatsu Y, Hioki K, Yamamoto M. Structural and functional alterations in the gut of parenterally or enterally fed rats. *J Surg Res* 1989; **47**: 129-133

62 **Koruda MJ**, Rolandelli RH, Settle RG, Zimmaro DM, Rombeau JL. Effect of parenteral nutrition supplemented with short-chain fatty acids on adaptation to massive small bowel resection. *Gastroenterology* 1988; **95**: 715-720

63 **Sukhotnik I**, Gork AS, Chen M, Drongowski RA, Coran AG, Harmon CM. Effect of a high fat diet on lipid absorption and fatty acid transport in a rat model of short bowel syndrome. *Pediatr Surg Int* 2003; **19**: 385-390

64 **Niot I**, Poirier H, Besnard P. Regulation of gene expression by fatty acids: special reference to fatty acid-binding protein (FABP). *Biochimie* 1997; **79**: 129-133

65 **Keelan M**, Cheeseman CI, Clandinin MT, Thomson AB. Intestinal morphology and transport after ileal resection in rat is modified by dietary fatty acids. *Clin Invest Med* 1996; **19**: 63-70

66 **Thiesen A**, Wild GE, Keelan M, Clandinin MT, Agellon LB, Thomson AB. Locally and systemically active glucocorticosteroids modify intestinal absorption of lipids in rats. *Lipids* 2002; **37**: 159-166

67 **Thiesen A**, Tappenden KA, McBurney MI, Clandinin MT, Keelan M, Thomson BK, Agellon L, Wild G, Thomson AB. Dietary lipids alter the effect of steroids on the uptake of lipids following intestinal resection in rats. *Dig Dis Sci* 2002; **47**: 1686-1696

68 **Poirier H**, Niot I, Monnot MC, Braissant O, Meunier-Durmort C, Costet P, Pineau T, Wahli W, Willson TM, Besnard P. Differential involvement of peroxisome-proliferator-activated receptors alpha and delta in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. *Biochem J* 2001; **355**: 481-488

69 **Huin C**, Corriveau L, Bianchi A, Keller JM, Collet P, Krémarik-Bouillaud P, Domenjoud L, Bécuwe P, Schohn H, Ménard D, Dauça M. Differential expression of peroxisome proliferator-activated receptors (PPARs) in the developing human fetal digestive tract. *J Histochem Cytochem* 2000; **48**: 603-611

70 **Thiesen AL**, Tappenden KA, McBurney MI, Clandinin MT, Keelan M, Thomson BK, Wild GE, Thomson AB. Dietary lipids alter the effect of steroids on the transport of glucose after intestinal resection: Part I. Phenotypic changes and expression of transporters. *J Pediatr Surg* 2003; **38**: 150-160

71 **Huwiler A**, Kolter T, Pfeilschifter J, Sandhoff K. Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta* 2000; **1485**: 63-99

72 **Hakomori S**, Igarashi Y. Functional role of glycosphingolipids in cell recognition and signaling. *J Biochem* 1995; **118**: 1091-1103

73 **Stern LE**, Erwin CR, Falcone RA, Huang FS, Kemp CJ, Williams JL, Warner BW. cDNA microarray analysis of adapting bowel after intestinal resection. *J Pediatr Surg* 2001; **36**: 190-195

74 **Erwin CR**, Falcone RA Jr, Stern LE, Kemp CJ, Warner BW. Analysis of intestinal adaptation gene expression by cDNA expression arrays. *JPEN J Parenter Enteral Nutr* 2000; **24**: 311-316

75 **Spector AA**, Yorek MA. Membrane lipid composition and cellular function. *J Lipid Res* 1985; **26**: 1015-1035

76 **Keelan M**, Wierzbicki A, Clandinin MT, Walker K, Thomson AB. Alterations in dietary fatty acid composition alter rat brush border membrane phospholipid fatty acid composition. *Diabetes Res* 1990; **14**: 165-170

77 **Alberts B**, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Steroid Hormones, Thyroid Hormones, Retinoids, and Vitamin D Bind to Intracellular Receptors that are Ligand-activated Gene Regulatory Proteins. In: Wilson J, Hunt T. Molecular Biology of the Cell. New York: Garland Publishing, 1994: 729-731

78 **Bertoli E**, Masserini M, Sonnino S, Ghidoni R, Cestaro B, Tettamanti G. Electron paramagnetic resonance studies on the fluidity and surface dynamics of egg phosphatidylcholine vesicles containing gangliosides. *Biochim Biophys Acta* 1981; **647**: 196-202

79 **Meddings JB**, DeSouza D, Goel M, Thiesen S. Glucose transport and microvillus membrane physical properties along the crypt-villus axis of the rabbit. *J Clin Invest* 1990; **85**: 1099-1107

80 **Meddings JB**, Theisen S. Development of rat jejunum: lipid permeability, physical properties, and chemical composition. *Am J Physiol* 1989; **256**: G931-G940

81 **Meddings JB**. Lipid permeability of the intestinal microvillus membrane may be modulated by membrane fluidity in the rat. *Biochim Biophys Acta* 1989; **984**: 158-166

82 **Simons K**, Ikonen E. Functional rafts in cell membranes. *Nature* 1997; **387**: 569-572

83 **Anderson RG**. The caveolae membrane system. *Annu Rev Biochem* 1998; **67**: 199-225

84 **Brown DA**, London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* 1998; **14**: 111-136

85 **Galbiati F**, Razani B, Lisanti MP. Emerging themes in lipid rafts and caveolae. *Cell* 2001; **106**: 403-411

86 **Neo CC**. "Effect of ganglioside-supplemented diets on the absorption of sugars and lipids in weanling rats" MSc thesis, University of Alberta, 2002

87 **Park EJ**, Suh M, Ramanujam K, Steiner K, Begg D, Clandinin MT. Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *J Pediatr Gastroenterol Nutr*

2005; **40**: 487-495
88 **Park EJ**, Suh M, Thomson B, Thomson AB, Ramanujam KS, Clandinin MT. Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology* 2005; **15**: 935-942
89 **Runembert I**, Queffeulou G, Federici P, Vrtovsnik F, Colucci-Guyon E, Babinet C, Briand P, Trugnan G, Friedlander G, Terzi F. Vimentin affects localization and activity of sodium-glucose cotransporter SGLT1 in membrane rafts. *J Cell Sci* 2002; **115**: 713-724
90 **Ottlakan A**. Role of platelet-activating factor in glucose uptake and utilization of different tissues. *Eur Surg Res* 1998; **30**: 393-402
91 **Hardin J**, Kroeker K, Chung B, Gall DG. Effect of proinflammatory interleukins on jejunal nutrient transport. *Gut* 2000; **47**: 184-191
92 **García-Herrera J,** Navarro MA, Marca MC, de la Osada J, Rodríguez-Yoldi MJ. The effect of tumor necrosis factor-alpha on D-fructose intestinal transport in rabbits. *Cytokine* 2004; **25**: 21-30
93 **Park EJ**. "Influence of dietary ganglioside on neonatal development and gut protection by altering membrane lipid profiles in weanling rats" PhD thesis, University of Alberta, 2004
94 **Solberg DH**, Diamond JM. Comparison of different dietary sugars as inducers of intestinal sugar transporters. *Am J Physiol* 1987; **252**: G574-G584
95 **Ferraris RP**, Diamond J. Crypt-villus site of glucose transporter induction by dietary carbohydrate in mouse intestine. *Am J Physiol* 1992; **262**: G1069-G1073
96 **Shu R**, David ES, Ferraris RP. Dietary fructose enhances intestinal fructose transport and GLUT5 expression in weaning rats. *Am J Physiol* 1997; **272**: G446-G453
97 **Brasitus TA**, Dudeja PK, Bolt MJ, Sitrin MD, Baum C. Dietary triacylglycerol modulates sodium-dependent D-glucose transport, fluidity and fatty acid composition of rat small intestinal brush-border membrane. *Biochim Biophys Acta* 1989; **979**: 177-186
98 **Cheeseman CI**, Harley B. Adaptation of glucose transport across rat enterocyte basolateral membrane in response to altered dietary carbohydrate intake. *J Physiol* 1991; **437**: 563-575
99 **Thomson AB**, Valberg LS. Kinetics of intestinal iron absorption in the rat: effect of cobalt. *Am J Physiol* 1971; **220**: 1080-1085
100 **Thomson AB**, Keelan M, Clandinin MT, Rajotte RV, Cheeseman C, Walker K. Use of polyunsaturated fatty acid diet to treat the enhanced intestinal uptake of lipids in streptozotocin diabetic rats. *Clin Invest Med* 1988; **11**: 57-61
101 **Thomson AB**, Keelan M, Clandinin MT. Feeding rats a diet enriched with saturated fatty acids prevents the inhibitory effects of acute and chronic ethanol exposure on the in vitro uptake of hexoses and lipids. *Biochim Biophys Acta* 1991; **1084**: 122-128
102 **Vine DF**, Charman SA, Gibson PR, Sinclair AJ, Porter CJ. Effect of dietary fatty acids on the intestinal permeability of marker drug compounds in excised rat jejunum. *J Pharm Pharmacol* 2002; **54**: 809-819
103 **Massimino SP**, McBurney MI, Field CJ, Thomson AB, Keelan M, Hayek MG, Sunvold GD. Fermentable dietary fiber increases GLP-1 secretion and improves glucose homeostasis despite increased intestinal glucose transport capacity in healthy dogs. *J Nutr* 1998; **128**: 1786-1793
104 **Tappenden KA**, Thomson AB, Wild GE, McBurney MI. Short-chain fatty acid-supplemented total parenteral nutrition enhances functional adaptation to intestinal resection in rats. *Gastroenterology* 1997; **112**: 792-802
105 **Tappenden KA**, McBurney MI. Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. *Dig Dis Sci* 1998; **43**: 1526-1536
106 **Tappenden KA**, Drozdowski LA, Thomson AB, McBurney MI. Short-chain fatty acid-supplemented total parenteral nutrition alters intestinal structure, glucose transporter 2 (GLUT2) mRNA and protein, and proglucagon mRNA abundance in normal rats. *Am J Clin Nutr* 1998; **68**: 118-125
107 **Bartholome AL**, Albin DM, Baker DH, Holst JJ, Tappenden KA. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. *JPEN J Parenter Enteral Nutr* 2004; **28**: 210-22; discussion 222-3
108 **Scharrer E**. Adaptation of intestinal amino acid transport. *Experientia* 1972; **28**: 267
109 **Lis MT**, Crampton RF, Matthews DM. Effect of dietary changes on intestinal absorption of L-methionine and L-methionyl-L-methionine in the rat. *Br J Nutr* 1972; **27**: 159-167
110 **Casirola DM**, Vinnakota RR, Ferraris RP. Intestinal amino acid transport in mice is modulated by diabetes and diet. *J Nutr* 1994; **124**: 842-852
111 **Ziegler TR**, Mantell MP, Chow JC, Rombeau JL, Smith RJ. Gut adaptation and the insulin-like growth factor system: regulation by glutamine and IGF-I administration. *Am J Physiol* 1996; **271**: G866-G875
112 **Tamada H**, Nezu R, Matsuo Y, Imamura I, Takagi Y, Okada A. Alanyl glutamine-enriched total parenteral nutrition restores intestinal adaptation after either proximal or distal massive resection in rats. *JPEN J Parenter Enteral Nutr* 1993; **17**: 236-242
113 **Wirén ME**, Permert J, Skullman SP, Wang F, Larsson J. No differences in mucosal adaptive growth one week after intestinal resection in rats given enteral glutamine supplementation or deprived of glutamine. *Eur J Surg* 1996; **162**: 489-498
114 **Sukhotnik I**, Mogilner JG, Lerner A, Coran AG, Lurie M, Miselevich I, Shiloni E. Parenteral arginine impairs intestinal adaptation following massive small bowel resection in a rat model. *Pediatr Surg Int* 2005; **21**: 460-465
115 **Pegg AE**, McCann PP. Polyamine metabolism and function. *Am J Physiol* 1982; **243**: C212-C221
116 **Tabor CW**, Tabor H. 1,4-Diaminobutane (putrescine), spermidine, and spermine. *Annu Rev Biochem* 1976; **45**: 285-306
117 **Dall'Asta V**, Gazzola GC, Franchi-Gazzola R, Bussolati O, Longo N, Guidotti GG. Pathways of L-glutamic acid transport in cultured human fibroblasts. *J Biol Chem* 1983; **258**: 6371-6379
118 **Uda K**, Tsujikawa T, Ihara T, Fujiyama Y, Bamba T. Luminal polyamines upregulate transmural glucose transport in the rat small intestine. *J Gastroenterol* 2002; **37**: 434-441
119 **Czernichow B**, Nsi-Emvo E, Galluser M, Gossé F, Raul F. Enteral supplementation with ornithine alpha ketoglutarate improves the early adaptive response to resection. *Gut* 1997; **40**: 67-72
120 **Dumas F**, De Bandt JP, Colomb V, Le Boucher J, Coudray-Lucas C, Lavie S, Brousse N, Ricour C, Cynober L, Goulet O. Enteral ornithine alpha-ketoglutarate enhances intestinal adaptation to massive resection in rats. *Metabolism* 1998; **47**: 1366-1371
121 **Tappenden KA**, Thomson AB, Wild GE, McBurney MI. Short-chain fatty acids increase proglucagon and ornithine decarboxylase messenger RNAs after intestinal resection in rats. *JPEN J Parenter Enteral Nutr* 1996; **20**: 357-362
122 **Thompson JS**. Can the intestine adapt to a changing environment? *Gastroenterology* 1997; **113**: 1402-1405
123 **Ulshen MH**, Dowling RH, Fuller CR, Zimmermann EM, Lund PK. Enhanced growth of small bowel in transgenic mice overexpressing bovine growth hormone. *Gastroenterology* 1993; **104**: 973-980
124 **Taylor B**, Murphy GM, Dowling RH. Pituitary hormones and the small bowel: effect of hypophysectomy on intestinal adaptation to small bowel resection in the rat. *Eur J Clin Invest* 1979; **9**: 115-127
125 **Lund PK**. Molecular basis of intestinal adaptation: the role of the insulin-like growth factor system. *Ann N Y Acad Sci* 1998; **859**: 18-36
126 **Gillingham MB**, Dahly EM, Murali SG, Ney DM. IGF-I treatment facilitates transition from parenteral to enteral nutrition in rats with short bowel syndrome. *Am J Physiol Regul Integr Comp Physiol* 2003; **284**: R363-R371
127 **Benhamou PH**, Canarelli JP, Leroy C, De Boissieu D, Dupont C. Stimulation by recombinant human growth hormone of growth and development of remaining bowel after subtotal ileojejunectomy in rats. *J Pediatr Gastroenterol Nutr* 1994; **18**:

446-452
128 **Park JH**, Vanderhoof JA. Growth hormone did not enhance mucosal hyperplasia after small-bowel resection. *Scand J Gastroenterol* 1996; **31**: 349-354
129 **Ljungmann K**, Grofte T, Kissmeyer-Nielsen P, Flyvbjerg A, Vilstrup H, Tygstrup N, Laurberg S. GH decreases hepatic amino acid degradation after small bowel resection in rats without enhancing bowel adaptation. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G700-G706
130 **Iannoli P**, Miller JH, Ryan CK, Gu LH, Ziegler TR, Sax HC. Human growth hormone induces system B transport in short bowel syndrome. *J Surg Res* 1997; **69**: 150-158
131 **Biolo G, Iscra F**, Bosutti A, Toigo G, Ciocchi B, Geatti O, Gullo A, Guarnieri G. Growth hormone decreases muscle glutamine production and stimulates protein synthesis in hypercatabolic patients. *Am J Physiol Endocrinol Metab* 2000; **279**: E323-E332
132 **Gu Y**, Wu ZH. The anabolic effects of recombinant human growth hormone and glutamine on parenterally fed, short bowel rats. *World J Gastroenterol* 2002; **8**: 752-757
133 **Zhou X**, Li YX, Li N, Li JS. Glutamine enhances the gut-trophic effect of growth hormone in rat after massive small bowel resection. *J Surg Res* 2001; **99**: 47-52
134 **Lobie PE**, Breipohl W, Waters MJ. Growth hormone receptor expression in the rat gastrointestinal tract. *Endocrinology* 1990; **126**: 299-306
135 **Green H**, Morikawa M, Nixon T. A dual effector theory of growth-hormone action. *Differentiation* 1985; **29**: 195-198
136 **Byrne T**, Wilmore D. Does growth hormone and glutamine enhance bowel absorption? *Gastroenterology* 1998; **114**: 1110-1112
137 **Jeppesen PB**, Szkudlarek J, Høy CE, Mortensen PB. Effect of high-dose growth hormone and glutamine on body composition, urine creatinine excretion, fatty acid absorption, and essential fatty acids status in short bowel patients: a randomized, double-blind, crossover, placebo-controlled study. *Scand J Gastroenterol* 2001; **36**: 48-54
138 **Scolapio JS**, Camilleri M, Fleming CR, Oenning LV, Burton DD, Sebo TJ, Batts KP, Kelly DG. Effect of growth hormone, glutamine, and diet on adaptation in short-bowel syndrome: a randomized, controlled study. *Gastroenterology* 1997; **113**: 1074-1081
139 **Byrne TA**, Persinger RL, Young LS, Ziegler TR, Wilmore DW. A new treatment for patients with short-bowel syndrome. Growth hormone, glutamine, and a modified diet. *Ann Surg* 1995; **222**: 243-54; discussion 254-5
140 **Messing** B, Pigot F, Rongier M, Morin MC, Ndeïndoum U, Rambaud JC. Intestinal absorption of free oral hyperalimentation in the very short bowel syndrome. *Gastroenterology* 1991; **100**: 1502-1508
141 **Gu Y**, Wu ZH, Xie JX, Jin DY, Zhuo HC. Effects of growth hormone (rhGH) and glutamine supplemented parenteral nutrition on intestinal adaptation in short bowel rats. *Clin Nutr* 2001; **20**: 159-166
142 **Thissen JP**, Ketelslegers JM, Underwood LE. Nutritional regulation of the insulin-like growth factors. *Endocr Rev* 1994; **15**: 80-101
143 **Seguy D**, Vahedi K, Kapel N, Souberbielle JC, Messing B. Low-dose growth hormone in adult home parenteral nutrition-dependent short bowel syndrome patients: a positive study. *Gastroenterology* 2003; **124**: 293-302
144 **Matarese LE**, Seidner DL, Steiger E. Growth hormone, glutamine, and modified diet for intestinal adaptation. *J Am Diet Assoc* 2004; **104**: 1265-1272
145 **Lemmey AB**, Ballard FJ, Martin AA, Tomas FM, Howarth GS, Read LC. Treatment with IGF-I peptides improves function of the remnant gut following small bowel resection in rats. *Growth Factors* 1994; **10**: 243-252
146 **Gillingham MB**, Kritsch KR, Murali SG, Lund PK, Ney DM. Resection upregulates the IGF-I system of parenterally fed rats with jejunocolic anastomosis. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1158-G1168
147 **Dahly EM**, Gillingham MB, Guo Z, Murali SG, Nelson DW, Holst JJ, Ney DM. Role of luminal nutrients and endogenous GLP-2 in intestinal adaptation to mid-small bowel resection. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G670-G682
148 **Knott AW**, Juno RJ, Jarboe MD, Profitt SA, Erwin CR, Smith EP, Fagin JA, Warner BW. Smooth muscle overexpression of IGF-I induces a novel adaptive response to small bowel resection. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G562-G570
149 **Opleta-Madsen K**, Meddings JB, Gall DG. Epidermal growth factor and postnatal development of intestinal transport and membrane structure. *Pediatr Res* 1991; **30**: 342-350
150 **Millar GA**, Hardin JA, Johnson LR, Gall DG. The role of PI 3-kinase in EGF-stimulated jejunal glucose transport. *Can J Physiol Pharmacol* 2002; **80**: 77-84
151 **Chung BM**, Wallace LE, Winkfein RK, O'Loughlin EV, Hardin JA, Gall DG. The effect of massive small bowel resection and oral epidermal growth factor therapy on SGLT-1 distribution in rabbit distal remnant. *Pediatr Res* 2004; **55**: 19-26
152 **Shin CE**, Falcone RA Jr, Duane KR, Erwin CR, Warner BW. The distribution of endogenous epidermal growth factor after small bowel resection suggests increased intestinal utilization during adaptation. *J Pediatr Surg* 1999; **34**: 22-26
153 **Hardin JA**, Chung B, O'loughlin EV, Gall DG. The effect of epidermal growth factor on brush border surface area and function in the distal remnant following resection in the rabbit. *Gut* 1999; **44**: 26-32
154 **O'Brien DP**, Nelson LA, Williams JL, Kemp CJ, Erwin CR, Warner BW. Selective inhibition of the epidermal growth factor receptor impairs intestinal adaptation after small bowel resection. *J Surg Res* 2002; **105**: 25-30
155 **Wong WM**, Wright NA. Epidermal growth factor, epidermal growth factor receptors, intestinal growth, and adaptation. *JPEN J Parenter Enteral Nutr* 1999; **23**: S83-S88
156 **Avissar NE**, Wang HT, Miller JH, Iannoli P, Sax HC. Epidermal growth factor receptor is increased in rabbit intestinal brush border membrane after small bowel resection. *Dig Dis Sci* 2000; **45**: 1145-1152
157 **Knott AW**, Erwin CR, Profitt SA, Juno RJ, Warner BW. Localization of postresection EGF receptor expression using laser capture microdissection. *J Pediatr Surg* 2003; **38**: 440-445
158 **Sham J**, Martin G, Meddings JB, Sigalet DL. Epidermal growth factor improves nutritional outcome in a rat model of short bowel syndrome. *J Pediatr Surg* 2002; **37**: 765-769
159 **Chung BM**, Wallace LE, Hardin JA, Gall DG. The effect of epidermal growth factor on the distribution of SGLT-1 in rabbit jejunum. *Can J Physiol Pharmacol* 2002; **80**: 872-878
160 **Nakai K**, Hamada Y, Kato Y, Kitagawa K, Hioki K, Ito S, Okumura T. Further evidence that epidermal growth factor enhances the intestinal adaptation following small bowel transplantation. *Life Sci* 2004; **75**: 2091-2102
161 **Yang H**, Wildhaber BE, Teitelbaum DH. 2003 Harry M. Vars Research Award. Keratinocyte growth factor improves epithelial function after massive small bowel resection. *JPEN J Parenter Enteral Nutr* 2003; **27**: 198-206; discussion 206-7
162 **Jéquier E**. Leptin signaling, adiposity, and energy balance. *Ann N Y Acad Sci* 2002; **967**: 379-388
163 **Campfield LA**, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 1995; **269**: 546-549
164 **Zhang Y**, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425-432
165 **Pearson PY**, O'Connor DM, Schwartz MZ. Novel effect of leptin on small intestine adaptation. *J Surg Res* 2001; **97**: 192-195
166 **Wren AM**, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR. Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 2001; **50**: 2540-2547
167 **Topstad D**, Martin G, Sigalet D. Systemic GLP-2 levels do not limit adaptation after distal intestinal resection. *J Pediatr Surg* 2001; **36**: 750-754
168 **Thulesen J**, Hartmann B, Kissow H, Jeppesen PB, Orskov C, Holst JJ, Poulsen SS. Intestinal growth adaptation and gluca-

gon-like peptide 2 in rats with ileal--jejunal transposition or small bowel resection. *Dig Dis Sci* 2001; **46**: 379-388
169 **Ljungmann K**, Hartmann B, Kissmeyer-Nielsen P, Flyvbjerg A, Holst JJ, Laurberg S. Time-dependent intestinal adaptation and GLP-2 alterations after small bowel resection in rats. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G779-G785
170 **Martin GR**, Wallace LE, Hartmann B, Holst JJ, Demchyshyn L, Toney K, Sigalet DL. Nutrient-stimulated GLP-2 release and crypt cell proliferation in experimental short bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G431-G438
171 **Perez A**, Duxbury M, Rocha FG, Ramsanahie AP, Farivar RS, Varnholt H, Ito H, Wong H, Rounds J, Zinner MJ, Whang EE, Ashley SW. Glucagon-like peptide 2 is an endogenous mediator of postresection intestinal adaptation. *JPEN J Parenter Enteral Nutr* 2005; **29**: 97-101
172 **Scott RB**, Kirk D, MacNaughton WK, Meddings JB. GLP-2 augments the adaptive response to massive intestinal resection in rat. *Am J Physiol* 1998; **275**: G911-G921
173 **Martin GR**, Wallace LE, Sigalet DL. Glucagon-like peptide-2 induces intestinal adaptation in parenterally fed rats with short bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G964-G972
174 **Washizawa N**, Gu LH, Gu L, Openo KP, Jones DP, Ziegler TR. Comparative effects of glucagon-like peptide-2 (GLP-2), growth hormone (GH), and keratinocyte growth factor (KGF) on markers of gut adaptation after massive small bowel resection in rats. *JPEN J Parenter Enteral Nutr* 2004; **28**: 399-409
175 **Jeppesen PB**, Hartmann B, Thulesen J, Graff J, Lohmann J, Hansen BS, Tofteng F, Poulsen SS, Madsen JL, Holst JJ, Mortensen PB. Glucagon-like peptide 2 improves nutrient absorption and nutritional status in short-bowel patients with no colon. *Gastroenterology* 2001; **120**: 806-815
176 **Jeppesen PB**. Clinical significance of GLP-2 in short-bowel syndrome. *J Nutr* 2003; **133**: 3721-3724
177 **Dodson BD**, Wang JL, Swietlicki EA, Rubin DC, Levin MS. Analysis of cloned cDNAs differentially expressed in adapting remnant small intestine after partial resection. *Am J Physiol* 1996; **271**: G347-G356
178 **Sugawara S**, Takeda K, Lee A, Dennert G. Suppression of stress protein GRP78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. *Cancer Res* 1993; **53**: 6001-6005
179 **Potten CS**, Merritt A, Hickman J, Hall P, Faranda A. Characterization of radiation-induced apoptosis in the small intestine and its biological implications. *Int J Radiat Biol* 1994; **65**: 71-78
180 **Rubin DC**, Swietlicki EA, Wang JL, Levin MS. Regulation of PC4/TIS7 expression in adapting remnant intestine after resection. *Am J Physiol* 1998; **275**: G506-G513
181 **Rountree DB**, Ulshen MH, Selub S, Fuller CR, Bloom SR, Ghatei MA, Lund PK. Nutrient-independent increases in proglucagon and ornithine decarboxylase messenger RNAs after jejunoileal resection. *Gastroenterology* 1992; **103**: 462-468
182 **Unmack MA**, Rangachari PK, Skadhauge E. Effects of isoprostanes and prostanoids on porcine small intestine. *J Pharmacol Exp Ther* 2001; **296**: 434-441
183 **Izadpanah A**, Dwinell MB, Eckmann L, Varki NM, Kagnoff MF. Regulated MIP-3alpha/CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G710-G719
184 **Murase T**, Kondo H, Hase T, Tokimitsu I, Saito M. Abundant expression of uncoupling protein-2 in the small intestine: up-regulation by dietary fish oil and fibrates. *Biochim Biophys Acta* 2001; **1530**: 15-22
185 **Balasubramaniam A**, Tao Z, Zhai W, Stein M, Sheriff S, Chance WT, Fischer JE, Eden PE, Taylor JE, Liu CD, McFadden DW, Voisin T, Roze C, Laburthe M. Structure-activity studies including a Psi(CH(2)-NH) scan of peptide YY (PYY) active site, PYY(22-36), for interaction with rat intestinal PYY receptors: development of analogues with potent in vivo activity in the intestine. *J Med Chem* 2000; **43**: 3420-3427
186 **Puolakkainen P**, Reed M, Vento P, Sage EH, Kiviluoto T, Kivilaakso E. Expression of SPARC (secreted protein, acidic and rich in cysteine) in healing intestinal anastomoses and short bowel syndrome in rats. *Dig Dis Sci* 1999; **44**: 1554-1564
187 **Vachon PH**, Cardin E, Harnois C, Reed JC, Plourde A, Vézina A. Early acquisition of bowel segment-specific Bcl-2 homolog expression profiles during development of the human ileum and colon. *Histol Histopathol* 2001; **16**: 497-510
188 **Nankervis CA**, Dunaway DJ, Miller CE. Endothelin ET(A) and ET(B) receptors in postnatal intestine. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G555-G562
189 **Juul SE**, Ledbetter DJ, Joyce AE, Dame C, Christensen RD, Zhao Y, DeMarco V. Erythropoietin acts as a trophic factor in neonatal rat intestine. *Gut* 2001; **49**: 182-189
190 **Jonas CR**, Farrell CL, Scully S, Eli A, Estívariz CF, Gu LH, Jones DP, Ziegler TR. Enteral nutrition and keratinocyte growth factor regulate expression of glutathione-related enzyme messenger RNAs in rat intestine. *JPEN J Parenter Enteral Nutr* 2000; **24**: 67-75
191 **Kato Y**, Yu D, Schwartz MZ. Enhancement of intestinal adaptation by hepatocyte growth factor. *J Pediatr Surg* 1998; **33**: 235-239
192 **Sacks AI**, Warwick GJ, Barnard JA. Early proliferative events following intestinal resection in the rat. *J Pediatr Gastroenterol Nutr* 1995; **21**: 158-164
193 **Goyal A**, Singh R, Swietlicki EA, Levin MS, Rubin DC. Characterization of rat epimorphin/syntaxin 2 expression suggests a role in crypt-villus morphogenesis. *Am J Physiol* 1998; **275**: G114-G124
194 **Bernal NP**, Stehr W, Zhang Y, Profitt S, Erwin CR, Warner BW. Evidence for active Wnt signaling during postresection intestinal adaptation. *J Pediatr Surg* 2005; **40**: 1025-1029; discussion 1029
195 **Jao W**, Sileri P, Holaysan J, Morini S, Chejfec G, Rastellini C, Benedetti E, Cicalese L. Morphologic adaptation following segmental living related intestinal transplantation. *Transplant Proc* 2002; **34**: 924
196 **Benedetti E**, Baum C, Cicalese L, Brown M, Raofi V, Massad MG, Abcarian H. Progressive functional adaptation of segmental bowel graft from living related donor. *Transplantation* 2001; **71**: 569-571

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REVIEW

Novel strategies for the treatment of inflammatory bowel disease: Selective inhibition of cytokines and adhesion molecules

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Abstract

The etiology of inflammatory bowel disease (IBD) has not yet been clarified and immunosuppressive agents which non-specifically reduce inflammation and immunity have been used in the conventional therapies for IBD. Evidence indicates that a dysregulation of mucosal immunity in the gut of IBD causes an overproduction of inflammatory cytokines and trafficking of effector leukocytes into the bowel, thus leading to an uncontrolled intestinal inflammation. Such recent advances in the understanding of the pathogenesis of IBD created a recent trend of novel biological therapies which specifically inhibit the molecules involved in the inflammatory cascade. Major targets for such treatment are inflammatory cytokines and their receptors, and adhesion molecules. A chimeric anti-TNF-α monoclonal antibody, infliximab, has become a standard therapy for CD and it is also likely to be beneficial for UC. Several anti-TNF reagents have been developed but most of them seem to not be as efficacious as infliximab. A humanized anti-TNF monoclonal antibody, adalimumab may be useful for the treatment of patients who lost responsiveness or developed intolerance to infliximab. Antibodies against IL-12 p40 and IL-6 receptor could be alternative new anti-cytokine therapies for IBD. Anti-interferon-γ and anti-CD25 therapies were developed, but the benefit of these agents has not yet been established. The selective blocking of migration of leukocytes into intestine seems to be a nice approach. Antibodies against α4 integrin and α4β7 integrin showed benefit for IBD. Antisense oligonucleotide of intercellular adhesion molecule 1 (ICAM-1) may be efficacious for IBD. Clinical trials of such compounds have been either recently reported or are currently underway. In this article, we review the efficacy and safety of such novel biological therapies for IBD.

INTRODUCTION

Although the etiology of inflammatory bowel disease (IBD), such as, Crohn's disease (CD) and ulcerative colitis (UC), has not yet been fully addressed, there has been remarkable progress in the understanding of this field in the past decade. In normal bowels, the immune reaction is sophisticatedly regulated while keeping a balance between the effectors and the regulators, and as a result, the homeostasis of the gut is maintained. A lot of evidences indicate that mucosal immunity is dysregulated in the bowel of IBD[1]. Two forms of IBD show distinct profiles of T cell mediated immunity. In the gut of CD, a strong Th1 reaction is induced, while the Th2 response is upregulated in the colon of UC[2]. Particularly in CD, it is evident that Th1 dominant immunity plays an important role in the pathogenesis. In UC, although the relevance of elevated Th2 cytokines to the colonic inflammation has not yet been clarified, an increased proinflammatory cytokine production is also observed which seems to be related to the inflammation. For the development of intestinal inflammation, leukocyte trafficking to the gut is an important step[3]. Adhesion molecules, such as integrins, mediate the selective binding between the leukocytes and the endothelial cells and thus the migration of leukocytes into the normal and inflamed intestine. The main classical medical treatments for IBD are steroids and immunosuppressive agents which non-specifically reduce immunity and inflammation. Recent advances in the understanding of the mechanism of bowel inflammation have led to a recent trend in the development of biological therapies which selectively inhibit the action of molecules essential to the inflammatory process. Major targets for such therapies are inflammatory cytokines and their receptors, and adhesion molecules. Re-

cently, numerous challenges have been performed to generate anti-cytokine and anti-integrin compounds to treat IBD. This article reviews the efficacy and safety of such novel biological therapies for IBD targeting cytokines, cytokine receptors and adhesion molecules.

INHIBITION OF TNF

TNF-α is a proinflammatory cytokine which is abundantly expressed in the gut of CD[4-6]. In animal models of experimental colitis, treatment with anti-TNF-α antibody has been shown to be effective in the suppression of intestinal inflammation[7,8]. As a result, this cytokine was considered to be an attractive target for the treatment of IBD and several anti-TNF reagents have thus been developed. These reagents include infliximab, CDP571, CDP870, etanercept, onercept and adalimumab. Infliximab is a chimeric IgG1 monoclonal antibody against TNF-α, which was created in late 1980s, and it has been demonstrated to be effective in reducing intestinal inflammation in CD as described below. Most of the other anti-TNF reagents are modified by a reduction of the mouse peptide sequence or are completely humanized in order to reduce the immunogenecity. Not all of the other anti-TNF reagents have been proven to be as effective in the treatment of CD as infliximab, and the efficacy of such reagents seems to be dependent, not only on the ability to neutralize soluble TNF-α, but also on the capacity to bind to the membrane-bound TNF-α on the cell surface, thereby mediating the apoptosis of the effector cells[9].

Infliximab

Infliximab was demonstrated to be effective in both the induction and maintenance therapy for refractory luminal and fistulizing CD. In a randomized double-blind placebo-controlled trial, 108 patients with moderate-to-severe CD which is resistant to conventional therapy, were treated with the single intravenous infusion of either placebo or infliximab at a dose of 5 mg/kg, 10 mg/kg or 20 mg/kg. The rates of the clinical response at 4 wk were 81% for infliximab 5 mg/kg, 50% for infliximab 10 mg/kg and 64% for infliximab 20 mg/kg, all of which were significantly higher than that for the placebo-treated group. The clinical remission rate at 4 wk was also significantly higher in the infliximab-treated group than that in the placebo-treated group (33% *vs* 4%)[10]. In a randomized, double-blind, placebo-controlled trial for the treatment of fistulizing disease, 94 CD patients with draining abdominal and perianal fistulas refractory to conventional therapy were treated with three intravenous infusions at wk 0, 2 and 6 of either a placebo or infliximab at a dose of 5 mg/kg or 10 mg/kg. The response rates were significantly greater in the infliximab 5 mg/kg group (68%) and in the infliximab 10 mg/kg group (56%) than that in the placebo-treated group (26%). The rates of a complete closure of the fistulas were also significantly higher in the infliximab 5 mg/kg group (55%) and in the infliximab 10 mg/kg group (38%) than in the placebo-treated group (13%)[11]. The effectiveness of infliximab for the maintenance therapy for inflammatory CD was assessed in a large trial called ACCENT I. Three hundred and thirty-five responders to a single infusion of infliximab were subsequently treated with 5 mg/kg infliximab at wk 2 and 6, followed by infusions of either 5 mg/kg or 10 mg/kg infliximab once every 8 wk until wk 54, or they were treated with placebo at wk 2 and 6, and subsequently every 8 wk. The rates of clinical response and remission at wk 30 and 54 was significantly greater in both groups receiving 5 mg/kg and 10 mg/kg infliximab every 8 wk than those in the placebo-treated group[12]. In addition, an analysis comparing the scheduled and episodic treatment strategies of infliximab for CD was conducted based on the ACCENT I data. The efficacy of the scheduled therapy was better than episodic strategy in terms of CDAI score, clinical remission and response rates, improvement in IBDQ score, mucosal healing and CD-related hospitalization and surgery[13]. For an evaluation of the infliximab maintenance therapy for fistulizing CD, ACCENT II trial was conducted. One hundred and ninety-six CD patients with draining perianal and enterocutaneous fistulas who responded to the induction therapy with three infusions of 5 mg/kg infliximab at wk 0, 2 and 6 received either a placebo or 5 mg/kg infliximab every 8 wk. The median time to the loss of response, response rate and complete fistula closure rate at wk 54 in the infliximab maintenance group were significantly greater than those in the placebo group[14].

Regarding the safety of infliximab treatment, it is well tolerated in the majority of the patients. In randomized controlled clinical trials, the rates of adverse events occurring in infliximab-treated patients were comparable to those in placebo-treated patients[10-12,14]. Serious side effects, however, have been reported and attention must be paid to the possible occurrence of serious infections and autoimmune disorders, as well as the theoretical threat of cancer and lymphoma. In an analysis of 500 infliximab-treated patients in Mayo Clinic, serious adverse events were observed in 8.6%, of which 6% was considered to possibly be related to infliximab[15]. Such events included serious infections, severe infusion reactions, serum sickness-like reactions, drug-induced lupus, cancer, non-Hodgkin's lymphoma and demyelinating process. The infectious complications included fatal sepsis, pneumonia, viral gastroenteritis, abdominal abscesses requiring surgery and histoplasmosis. Five deaths (1%) were observed which were likely or possibly related to infliximab. The reactivation of latent tuberculosis has been reported elsewhere[16], as a result, it is recommended that all patients be screened for latent tuberculosis before the initiation of this treatment regimen.

Infliximab is a mouse/human chimeric monoclonal antibody of which 25% is mouse peptide sequence. The murine component is ascribed to its immunogenecity, such as infusion-related reactions and serum sickness-like diseases. In such immunological reactions, the formation of antibodies against infliximab, called human anti-chimeric antibodies (HACA) is of particular concern as the presence of HACA is associated with an increased frequency of infusion reactions and the reduction in efficacy[17]. Concomitant immunosuppressive therapy and premedication with 200 mg of hydrocortisone reduce the frequency of HACA formation[17,18]. The scheduled infusions in the maintenance therapy have been shown to be associated

with the reduction of the rate of HACA formation[13]. As a result, the regular infliximab-treatment every 8 wk is likely to be beneficial for CD patients, not only for the maintenance of the remission state but also for the avoidance of infusion reactions.

The efficacy of infliximab for the treatment of UC remains controversial as two randomized controlled trials for steroid-refractory UC resulted in opposite results. A study indicated the benefit of infliximab for UC since 50% (4 of 8 patients) of the infliximab-treated patients showed treatment success, while none of the 3 patients receiving a placebo showed response[19]. However, this study was terminated prematurely because of a slow enrollment. Another study failed to show any benefit of infliximab over placebo as there was no significant difference between patients who received infliximab and placebo in the remission rates and an improvement in the activity scores[20]. Recently, two large multicenter randomized trials, ACT1 and ACT2, have been performed. In both trials, 364 patients with active UC were randomized to receive placebo or infliximab in a dose of 5 mg/kg or 10 mg/kg at wk 0, 2, 6, 14 and 22 in ACT1, and at wk 0, 2 and 6 then every 8 wk through wk 46 in ACT2. In both trials, both 5 mg/kg and 10 mg/kg infliximab showed significantly greater percentages in both the induction and maintenance of clinical remission and response, and in mucosal healing than placebo at both wk 8 and 30[21,22]. In addition, it was also recently demonstrated that infliximab is effective as a rescue therapy to avoid a colectomy or death in severe to moderately severe UC refractory to conventional therapies[23]. As a result, infliximab thus appears to also be efficacious for the treatment of UC as well as for CD.

CDP571

CDP571 is a "humanized" IgG4 antibody against TNF-α, created by genetic engineering to replace the murine component other than the binding domain with parts of a human IgG4 molecule. The resulting molecule is a chimera of 95% human and 5% mouse residues. The first study of 31 patients with active CD demonstrated that CDP571 5 mg/kg resulted in a greater decrease in the mean CDAI score at wk 2 compared with placebo[24]. After a promising pilot trial, CDP571 was tested in a placebo-controlled dose-finding trial[25]. In this study, 169 patients were randomized to receive a single intravenous infusion of either CDP571 in a dose of 10 or 20 mg/kg, or placebo. At wk 2, the clinical response rate was significantly higher in the patients treated with CDP571 (45%) than in those received a placebo (27%). Re-treatment was performed either every 8 wk with a placebo or CDP571 10 mg/kg, or every 12 wk with a placebo or CDP571 10 mg/kg (4 groups). The clinical remission rates at wk 24 in CDP571-treated groups were not significantly different from those of the placebo-treated groups. In a subsequent, randomized, double-blind, placebo-controlled, multicenter study[26], the efficacy and tolerability of CDP571 in 396 patients with active CD was evaluated. Among the patients treated with CDP571 10 mg/kg every 8 wk, the percentage of patients achieving a clinical response was significantly higher than in those receiving a placebo at wk 2 and 4. However, at wk 28 the difference was not statistically significant. As a result, CDP571 therapy showed a short term benefit in induction therapy, but it is not sufficient to maintain a long term effect. In a post-hoc exploratory analysis of a subgroup of patients with elevated baseline CRP levels, there was a significant difference in the number of patients showing a clinical response at wk 2 (CDP571, 49.5%; placebo, 15.5%), and at all time points from wk 12 to wk 28, thus leaving the possibility that CDP571 is more efficacious in a selected group of patients[26]. CDP571 failed to show a steroid sparing effect in patients with steroid-dependent CD[27]. CDP571 was well tolerated even in patients with CD who developed either infusion reactions or delayed-type hypersensitivity reactions to infliximab[28]. From these results, CDP571 was considered to be safe but not as effective as infliximab for CD and further clinical development of this antibody for the treatment of CD has thus been discontinued.

CDP870

CDP870 is a pegylated Fab fragment of humanized anti-TNF monoclonal antibody. In a placebo-controlled dose-finding study[29], 292 patients were randomized to receive a subcutaneous dose of CDP870 (100, 200, or 400 mg) or placebo at wk 0, 4, and 8. The group that received CDP870 400 mg showed greater clinical response rates than other groups at all time points. The clinical response rates of the CDP870 400 mg group were significantly higher than those of the placebo treated group at wk 2, 4, 8 and 10. The difference, however, did not reach statistical significance at wk 12. A greater dose separation was evident in the analysis of a patient subgroup with elevated CRP levels. In an exploratory analysis[30] in 119 patients with increased CRP levels ($\geqslant$ 10 mg/L), the differences in the clinical response between the 400 mg/dose (53.1%) and placebo (17.9%) were significant at 12 wk. These studies, therefore, indicated that CDP870 may be more effective in patients with elevated CRP levels. CDP870 seems to be safer and less immunogenic than infliximab. The efficacy, however, is likely to be lower than that of infliximab. The question arises whether physicians want to compromise on efficacy in the scope of better long-term safety. Further clinical trials are ongoing.

Etanercept

Etanercept is a genetically engineered fusion protein consisting of two recombinant human TNF p75 receptors linked to an Fc portion of human IgG1 fragment. The subcutaneous injection of etanercept at a dose of 25 mg twice weekly, which is an effective dose for rheumatoid arthritis, is a safe but ineffective dose for the treatment of patients with moderate to severe CD[31].

Onercept

Onercept, a recombinant, fully human, soluble p55 TNF receptor has showed efficacy to CD in an open-label pilot study (n = 12)[32]. A large, placebo-controlled, dose-finding study has been completed but the data have not yet been published. A press release by Serono (Geneve, Switzerland) revealed that the primary endpoint of this trial was not met.

Adalimumab

Adalimumab is a fully humanized anti-TNF monoclonal IgG1 antibody. This antibody is as efficacious as infliximab for the treatment of rheumatoid arthritis. *In vitro* studies revealed that this antibody is capable of inducing apoptosis in monocytes as infliximab[33]. As adalimumab does not contain a mouse peptide sequence, it is expected to be less immunogenic and more tolerable than infliximab. Two uncontrolled pilot studies of adalimumab with CD patients who had lost responsiveness or developed intolerance to infliximab[34,35] showed that subcutaneous adalimumab was well tolerated, thus suggesting a clinical benefit of adalimumab. In a phase 3, multicenter trial for active CD, clinical remission rates of patients who received adalimumab 160 mg at wk 0 and 80 mg at wk 2 was significantly higher than that of placebo at wk 4 (36% *vs* 12%)[36]. From these results, adalimumab is likely to be efficacious for the treatment of CD and it could thus be an alternative therapy for the patients who either lost responsiveness or developed intolerance to infliximab.

INHIBITION OF OTHER INFLAMMATORY CYTOKINES

Anti-IL-12 p40 antibody

IL-12, a heterodimeric molecule composed of IL-12 p40 and IL-12 p35 subunits, plays a central role in Th1 development. IL-12 is abundantly produced in the gut of CD patients[2]. In several animal models of Th1-mediated colitis, anti-IL-12 treatment effectively ameliorates intestinal inflammation[37,38]. IL-12 p40 subunit is also a component of another Th1 cytokine, IL-23, in which p40 forms a heterodimer with p19 subunit. IL-12 p40 is, therefore, a potential target for the treatment of CD in which intestinal inflammation is Th1-mediated. A double-blind, placebo-controlled randomized study of a humanized IgG1 monoclonal antibody against IL-12 p40 (ABT-874) was performed in 79 patients with active CD[39]. The patients were randomly assigned to receive seven weekly injections of 1 mg/kg anti-IL-12, 3 mg/kg anti-IL-12 or placebo subcutaneously either with or without 4 wk intervals between the first two injections. The patients who received 3 mg/kg anti-IL-12 for 7 wk showed a significantly greater clinical response rate than the patients treated with a placebo (75% *vs* 25%). The rates of remission were also higher in the 3 mg/kg anti-IL-12 group (38%) than in the placebo group (0%) but the difference did not reach statistical significance. The production of IL-12 and other Th1 and proinflammatory cytokines from patients' colonic lamina propria mononuclear cells dramatically decreased after the anti-IL-12 therapy. The most frequent adverse event was a local reaction at the injection site, which was observed with a greater rate in the anti-IL-12-treated group than in the placebo-treated group. Anti-drug antibody was formed in some patients who received anti-IL-12 antibody. No serious side effects requiring the discontinuation of the treatment, due to the anti-IL-12 therapy, were observed. Anti-IL-12 therapy is therefore considered to be a safe and effective treatment for active CD.

MRA (Anti-IL-6 receptor antibody)

IL-6 is one of the major inflammatory cytokines. IL-6 can transduce signals into cells without IL-6 receptor expression when IL-6 binds to soluble IL-6 receptor. The expression of IL-6 and soluble IL-6 receptor increases in patients with active CD[40,41]. A pilot randomized double-blind placebo-controlled trial of a humanized anti-IL-6 receptor monoclonal antibody, MRA, with active CD was performed[42]. Thirty-six patients were randomized biweekly to receive either a placebo, 8 mg/kg MRA or MRA/placebo alternately for 12 wk. The clinical remission rate with biweekly MRA was significantly higher than that with placebo (80% *vs* 31%). The acute phase responses such as ESR and CRP levels were rapidly suppressed 2 wk after the MRA injection. The incidence of adverse events was similar in all groups, and thus MRA treatment was generally well tolerated. It is, therefore, likely that anti-IL-6 receptor therapy is beneficial for active CD.

Fontolizumab (Anti-interferon-γ antibody)

Interferon-γ is a key cytokine that enhances the development of a Th1 immune response. Fontolizumab is a humanized monoclonal antibody directed against interferon-γ. A phase 2 study of fontolizumab at intravenous doses of 4 mg/kg or 10 mg/kg in 133 patients with moderate to severe active CD did not demonstrate efficacy at d 28. However, exploratory analyses based on 91 patients who received a second dose of fontolizumab at d 28 did demonstrate efficacy. This effect was most prominent in patients with elevated baseline concentrations of CRP[43]. An additional phase 2 study of fontolizumab at lower subcutaneous doses of 1.0 mg/kg or 4.0 mg/kg in 196 patients with active CD did not demonstrate efficacy at d 28[44]. These results indicate that a single dose may not be sufficient to achieve a significant improvement. Further clinical studies of fontolizumab for the induction and maintenance of remission in patients with CD are anticipated.

Anti-IL-2 receptor (CD25) antibodies

Daclizumab: IL-2 is a major T cell growth factor, which is secreted by activated T cells and acts via the high-affinity IL-2 receptor on T cells themselves to promote cell survival and proliferation. The IL-2 receptor α-chain (CD25) is a component of high-affinity IL-2 receptor and it is expressed on activated T cells. Daclizumab is a humanized monoclonal antibody to CD25, which blocks the binding of IL-2 to the IL-2 receptor. An open label pilot study of daclizumab suggested that it was beneficial for patients with active UC[45]. However, a recent placebo-controlled phase 2 trial of daclizumab at intravenous doses of 1 mg/kg twice with a 4-wk interval or 2 mg/kg every 2 wk for a total of four doses in 159 patients with active UC failed to show any efficacy[46].

Basiliximab: Basiliximab is a chimeric monoclonal antibody against CD25, which blocks the binding of IL-2 to the IL-2 receptor. Two uncontrolled pilot studies suggested that basiliximab in combination with steroids may be effective for steroid resistant UC[47,48]. A large random-

ized controlled trial is required to confirm the therapeutic benefit of this compound.

INHIBITION OF ADHESION MOLECULES

Many adhesion molecules play an important role in trafficking leukocytes into the inflamed gut wall and they are up-regulated in both CD and UC[49,50].

α4-integrins, predominantly expressed on lymphocytes, usually exist in combination with a β subunit and interact with adressins expressed on endothelium. α4β1-integrin binds to vascular cellular adhesion molecule 1 (VCAM-1) and α4β7-integrin binds to mucosal addressing cell adhesion molecule 1 (MAdCAM-1). The interaction between α4β7-integrin and MAdCAM-1 is important in mediating lymphocytes homing to the gut mucosa[51].

Leukocyte function-associated antigen 1 (LFA-1) expressed on leukocytes interacts with intercellular adhesion molecule 1 (ICAM-1), which is constitutively expressed at low levels on vascular endothelial cells and a subset of leukocytes, and they are up-regulated on many cell types in response to proinflammatory mediators[52].

Natalizumab

Natalizumab, a humanized IgG4 anti-α4-integrin monoclonal antibody, inhibits both α4β7-integrin/MAdCAM-1 interaction and α4β1/VCAM-1 binding.

In an initial small trial in 30 patients with active CD, a single 3 mg/kg intravenous infusion of natalizumab showed a short term effect in inducing remission at wk 2 and elevated circulating lymphocyte levels after the natalizumab infusion. Therefore, it is suggested that the natalizumab interrupted lymphocyte trafficking into the intestine[53]. In a large placebo-controlled randomized trial including 248 patients with moderate to severe CD, patients were treated twice at 4 wk intervals with 3 or 6 mg/kg of natalizumab or placebo. A significantly higher number of patients achieved remission at wk 6 only in the 3 + 3 mg/kg natalizumab group compared with the two infusions of placebo group (44% *vs* 27%). The clinical response rates at wk 6 in all treatment groups were significantly higher than that in the placebo-treated group (3 mg/kg natalizumab + placebo: 59%, 3 + 3 mg/kg natalizumab: 71%, 6 + 6 mg/kg natalizumab: 57% and the two infusions of placebo group, 38%)[54]. A larger phase 3 trial of ENACT-1 in 905 patients with moderate to severe CD failed to show a benefit for three intravenous infusions of 300 mg natalizumab every 4 wk. In a subgroup analysis, however, natalizumab-treated patients with concurrent immunosuppressive therapies, prior anti-TNF-α therapy or elevated CRP levels showed a significant response rate compared with placebo-treated patients[55]. Three hundred and thirty-nine patients with CD who responded to natalizumab in ENACT-1 were re-randomized to maintenance therapy with natalizumab (300 mg) or a placebo for up to 12 additional monthly infusions. In this maintenance study (ENACT-2), natalizumab demonstrated a significant superiority over the placebo in its ability to sustain both the response and remission at all consecutive time points over a 6-mo period and enabled patients to be successfully withdrawn from steroids[56]. In an uncontrolled short term pilot study in 10 patients with active UC, a single 3 mg/kg intravenous infusion of natalizumab showed a short term benefit[57].

Natalizumab is efficacious in multiple sclerosis (MS) as well[58]. In MS, α4β1 integrin/VCAM-1 binding appears to be a crucial step because antiα4β1 integrin antibody prevented the development of experimental autoimmune encephalomyelitis, a model of human MS[59]. Against these effects of natalizumab in IBD and MS, 3 patients receiving repeated treatment with natalizumab developed JC virus related progressive multifocal leukoencephalopathy (PML)[60-62]. PML, which almost invariably occurs in patients with AIDS or leukemia or in organ-transplant recipients, is a fatal opportunistic infection of the central nervous system caused by the reactivation of a clinically latent JC polyomavirus infection. Two patients with MS had been receiving the concomitant administration of interferon β-1a[60,61] and 1 patient with CD had been treated with natalizumab monotherapy[62]. These observations force us to reconsider both the efficacy and the potential risks associated with an inhibition of lymphocytes trafficking by anti-α4 integrin therapy.

MLN-02

MLN-02, a humanized anti-α4β7-integrin blocks specifically the α4β7-integrin/MAdCAM-1 interaction.

A randomized placebo-controlled trial in 185 patients with mild to moderately active CD treated with placebo, 0.5 mg/kg MLN-02 or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 57, 2.0 mg/kg MLN-02 showed significantly greater remission rates over the placebo (36.9% *vs* 20.7%). There was no significant difference between the actively treated and placebo-treated groups regarding the clinical response rates. No obvious differences in adverse events were noted among the three groups[63].

A randomized placebo-controlled trial in 181 patients with moderately active UC treated with placebo, 0.5 mg/kg MLN-02, or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 43 the remission rates were significantly higher in the actively treated groups (0.5 mg/kg: 33%, 2.0 mg/kg: 34%) than in the placebo-treated group (15%). An infusion reaction occurred in one MLN-02-treated patient who developed mild angioedema[64].

MLN-02 appears to be a generally well-tolerated and effective therapy especially for active UC, but further trials are necessary to confirm the efficacy of MLN-02 therapy for IBD.

Alicaforsen (ISIS 2302)

ISIS 2302 is a 20 base phosphorothioate oligodeoxynucleotide designed to specifically hybridize to the 3'-untranslated region of the human ICAM-1 mRNA. Treatment of ISIS 2302 *in vitro* resulted in a highly specific reduction in ICAM-1 mRNA and, consequently, a marked decrease in ICAM-1 protein expression[65]

A pilot trial in patients with moderate CD (including 15 patients treated with 13 intravenous infusions of 0.5, 1.0 or 2.0 mg/kg ISIS 2302 *vs* 5 patients with placebo over 26 d) demonstrated a higher remission rate in ISIS 2302-treated group compared with the placebo-treated group on d 33

(47% *vs* 20%)[66]. However, a placebo-controlled trial in 75 patients with steroid refractory CD failed to demonstrate efficacy was showed that the subcutaneous administration of ISIS 2302 induced only 3% of ISIS 2302-treated patients to clinical remission with complete steroid taper (0% in placebo-treated patients)[67]. Another larger randomized placebo-controlled trial also failed to show any benefit of ISIS 2302 for active CD[68]. Two hundred and ninety-nine patients with moderately active, steroid-dependent CD received placebo or ISIS 2302 (2 mg/kg intravenously three times a week) for 2 or 4 wk in mo 1 and 3. There were no differences in the steroid-free remission rates at wk 14 between the ISIS 2302-treated groups (2 wk: 20.2%, 4 wk: 21.2%) and the placebo-treated group (18.8%). However, a subgroup analysis using the area under the curve (AUC) of ISIS 2302 plasma concentration demonstrated an improvement of the clinical response for the highest AUC group (AUC > 65 μg × h/mL). This suggested that ISIS 2302 may be effective when given in adequate doses. As a result, a dose ranging pilot trial of high dose ISIS 2302 in 22 patients with active CD was conducted. The patients, who were infused with high dose ISIS 2302 (250 mg to 350 mg) intravenously three times a week for 4 wk, showed a 41% remission rate. Five patients, however, withdrew due to infusion-related symptoms[69].

A randomized placebo-controlled trial in 40 patients with mild to moderately active distal UC treated with 60 mL alicaforsen enema (0.1, 0.5, 2, or 4 mg/mL or placebo) once daily for 28 consecutive days showed a beneficial effect at the highest dose. Four mg/mL alicaforsen enema group showed a significant improvement in the disease activity index on d 29 and mo 3 in comparison to the placebo enema group[70]. An open-label, uncontrolled study in 12 patients with chronic unremitting pouchitis treated with 240 mg alicaforsen enema nightly for 6 wk demonstrated a significant improvement in the pouchitis disease activity index and an endoscopic mucosal appearance at wk 6[71].

The most consistently reported side effects of ISIS 2302 in all clinical trials were infusion reactions and a moderate increase in activated partial thromboplastin time.

CONCLUSION

Infliximab has changed the medical treatment of CD dramatically. This agent has been proven to be clearly effective for the induction and maintenance of remission for active CD meanwhile it is also generally well-tolerated. However, there are some patients who fail to respond to infliximab. In addition, some responders either lose responsiveness to infliximab during long-term therapy or develop intolerance to infliximab. The next issue will therefore be how to treat such patients who cannot be treated with infliximab. Adalimumab may be an alternative to infliximab. Anti-IL-12 and anti-IL-6 receptor therapies seem to be promising. The selective blocking of α4 integrin and α4β7 integrin also demonstrated promising results, however, the side effects still need to be fully elucidated. We await the results of further clinical trials to include such novel compounds in the algorithm for the treatment of CD. Infliximab is likely to also be beneficial for UC. In addition, some other agents seem to be effective for the treatment of UC and further clinical development is also underway. As a result, the management of UC may also dramatically improve in the near future owing to the use of such novel agents. The systematization of novel biological therapies for UC is therefore an issue that needs to be addressed in the future.

REFERENCES

1 **Strober W**, Nakamura K, Kitani A. The SAMP1/Yit mouse: another step closer to modeling human inflammatory bowel disease. *J Clin Invest* 2001; **107**: 667-670

2 **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**: 521-533

3 **Fiocchi C**. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; **115**: 182-205

4 **Murch SH**, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut* 1993; **34**: 1705-1709

5 **Breese EJ**, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, Walker-Smith JA, MacDonald TT. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* 1994; **106**: 1455-1466

6 **Braegger CP**, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* 1992; **339**: 89-91

7 **Powrie F**, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1994; **1:** 553-562

8 **Kosiewicz MM**, Nast CC, Krishnan A, Rivera-Nieves J, Moskaluk CA, Matsumoto S, Kozaiwa K, Cominelli F. Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. *J Clin Invest* 2001; **107**: 695-702

9 **Van den Brande JM**, Braat H, van den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, van Montfrans C, Hommes DW, Peppelenbosch MP, van Deventer SJ. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003; **124**: 1774-1785

10 **Targan SR**, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; **337**: 1029-1035

11 **Present DH**, Rutgeerts P, Targan S, Hanauer SB, Mayer L, van Hogezand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL, Schaible TF, van Deventer SJ. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999; **340**: 1398-1405

12 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549

13 **Rutgeerts P**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Hanauer SB. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004; **126**: 402-413

14 **Sands BE**, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, Kamm MA, Korzenik JR, Lashner BA, Onken JE, Rachmilewitz D, Rutgeerts P, Wild G, Wolf DC, Marsters PA, Travers SB, Blank MA, van Deventer SJ. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004; **350**: 876-885

15 **Colombel JF**, Loftus EV Jr, Tremaine WJ, Egan LJ, Harmsen WS, Schleck CD, Zinsmeister AR, Sandborn WJ. The safety profile of infliximab in patients with Crohn's disease: the Mayo clinic experience in 500 patients. *Gastroenterology* 2004; **126**: 19-31

16 **Keane J**, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 2001; **345**: 1098-1104

17 **Baert F**, Noman M, Vermeire S, Van Assche G, D' Haens G, Carbonez A, Rutgeerts P. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 2003; **348**: 601-608

18 **Farrell RJ**, Alsahli M, Jeen YT, Falchuk KR, Peppercorn MA, Michetti P. Intravenous hydrocortisone premedication reduces antibodies to infliximab in Crohn's disease: a randomized controlled trial. *Gastroenterology* 2003; **124**: 917-924

19 **Sands BE**, Tremaine WJ, Sandborn WJ, Rutgeerts PJ, Hanauer SB, Mayer L, Targan SR, Podolsky DK. Infliximab in the treatment of severe, steroid-refractory ulcerative colitis: a pilot study. *Inflamm Bowel Dis* 2001; **7:** 83-88

20 **Probert CS**, Hearing SD, Schreiber S, Kuhbacher T, Ghosh S, Arnott ID, Forbes A. Infliximab in moderately severe glucocorticoid resistant ulcerative colitis: a randomised controlled trial. *Gut* 2003; **52**: 998-1002

21 **Rutgeerts P**, Feagan BG, Olson A, Johanns J, Travers S, Present D, Sands BE, Sandborn W, Olson A. A randomized placebo-controlled trial of infliximab therapy for active ulcerative colitis: Act I trial. *Gastroenterology* 2005; **128:** A105

22 **Sandborn WJ**, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Olson A, Johanns J, Traverse S, Colombel JF. Infliximab induction and maintenance therapy for ulcerative colitis: the Act 2 trial. *Gastroenterology* 2005; **128:** A104-A105

23 **Järnerot G**, Hertervig E, Friis-Liby I, Blomquist L, Karlén P, Grännö C, Vilien M, Ström M, Danielsson A, Verbaan H, Hellström PM, Magnuson A, Curman B. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005; **128**: 1805-1811

24 **Stack WA,** Mann SD, Roy AJ, Heath P, Sopwith M, Freeman J, Holmes G, Long R, Forbes A, Kamm MA. Randomised controlled trial of CDP571 antibody to tumour necrosis factor-alpha in Crohn's disease. *Lancet* 1997; **349:** 521-524

25 **Sandborn WJ**, Feagan BG, Hanauer SB, Present DH, Sutherland LR, Kamm MA, Wolf DC, Baker JP, Hawkey C, Archambault A, Bernstein CN, Novak C, Heath PK, Targan SR. An engineered human antibody to TNF (CDP571) for active Crohn's disease: a randomized double-blind placebo-controlled trial. *Gastroenterology* 2001; **120**: 1330-1338

26 **Sandborn WJ**, Feagan BG, Radford-Smith G, Kovacs A, Enns R, Patel J. A randomized placebo-controlled trial of CDP571, a humanized monoclonal antibody to TNF-α in patients with moderate to severe Crohn's disease. *Gastroenterology* 2003; **124:** A61

27 **Feagan BG**, Feagan BG, Sandborn WJ, Baker JP, Cominelli F, Sutherland LR, Elson CD, Salzberg B, Archambault A, Bernstein CN, Lichtenstein GR, Heath PK, Hanauer SB. A randomized, double-blind, placebo-controlled, multi-center trial of the engineered human antibody to TNF (CDP571) for steroid sparing and maintenance of remission in patients with steroid-dependent Crohn's disease. *Gastroenterology* 2000; **118:** A655

28 **Hanauer S**, Present D, Targan SR, Kam L, Patel J, Sandborn WJ. CDP571, a humanized monoclonal antibody to TNF-a, is well tolerated in Crohn's disease patients with previous hypersensitivity to infliximab. *Gastroenterology* 2003; **124:** A517

29 **Schreiber S**, Rutgeerts P, Fedorak R, Klaliq-Kareemi M, Kamm MA, Patel J, the CDP870 CD Study Group. CDP870, a humanized anti-TNF antibody fragment induces clinical response with remission in patients with active Crohn's disease. *Gastroenterology* 2003; **124:** A61

30 **Schreiber S**, Fedorak R, Rutgeerts P, Innes A, Patel J. CDP870, a pegylated humanized anti-TNF antibody fragment, offers particular benefit to Crohn's disease patients with elevated C-reactive protein. *Gut* 2003; **52** Suppl 4: A4

31 **Sandborn WJ**, Hanauer SB, Katz S, Safdi M, Wolf DG, Baerg RD, Tremaine WJ, Johnson T, Diehl NN, Zinsmeister AR. Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2001; **121**: 1088-1094

32 **Rutgeerts P**, Lemmens L, Van Assche G, Noman M, Borghini-Fuhrer I, Goedkoop R. Treatment of active Crohn's disease with onercept (recombinant human soluble p55 tumour necrosis factor receptor): results of a randomized, open-label, pilot study. *Aliment Pharmacol Ther* 2003; **17**: 185-192

33 **Shen C**, Assche GV, Colpaert S, Maerten P, Geboes K, Rutgeerts P, Ceuppens JL. Adalimumab induces apoptosis of human monocytes: a comparative study with infliximab and etanercept. *Aliment Pharmacol Ther* 2005; **21:** 251-258

34 **Youdim A**, Vasiliauskas EA, Targan SR, Papadakis KA, Ippoliti A, Dubinsky MC, Lechago J, Paavola J, Loane J, Lee SK, Gaiennie J, Smith K, Do J, Abreu MT. A pilot study of adalimumab in infliximab-allergic patients. *Inflamm Bowel Dis* 2004; **10:** 333-338

35 **Sandborn WJ**, Hanauer S, Loftus EV Jr, Tremaine WJ, Kane S, Cohen R, Hanson K, Johnson T, Schmitt D, Jeche R. An open-label study of the human anti-TNF monoclonal antibody adalimumab in subjects with prior loss of response or intolerance to infliximab for Crohn's disease. *Am J Gastroenterol* 2004; **99:** 1984-1989

36 **Hanauer S**, Lukas M, Macintosh D, Rutgeerts P, Sandborn W, Pollack P. A randomized, double-blind, placebo-controlled trial of the human anti-TNF-α monoclonal antibody adalimumab for the induction of remission in patients with moderate to severely active Crohn's disease. *Gastroenterology* 2004; **127**: A332

37 **Neurath MF**, Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 1995; **182**: 1281-1290

38 **Davidson NJ**, Hudak SA, Lesley RE, Menon S, Leach MW, Rennick DM. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol* 1998; **161**: 3143-3149

39 **Mannon PJ**, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, Quezado M, Yang Z, Neurath MF, Salfeld J, Veldman GM, Schwertschlag U, Strober W. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 2004; **351**: 2069-2079

40 **Mitsuyama K**, Toyonaga A, Sasaki E, Ishida O, Ikeda H, Tsuruta O, Harada K, Tateishi H, Nishiyama T, Tanikawa K. Soluble interleukin-6 receptors in inflammatory bowel disease: relation to circulating interleukin-6. *Gut* 1995; **36**: 45-49

41 **Hosokawa T,** Kusugami K, Ina K, Ando T, Shinoda M, Imada A, Ohsuga M, Sakai T, Matsuura T, Ito K, Kaneshiro K. Interleukin-6 and soluble interleukin-6 receptor in the colonic mucosa of inflammatory bowel disease. *J Gastroenterol Hepatol* 1999; **14:** 987-996

42 **Ito H**, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, Matsumoto T, Yamamura T, Azuma J, Nishimoto N, Yoshizaki K, Shimoyama T, Kishimoto T. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 2004; **126**: 989-996; discussion 947

43 **Hommes D,** Mikhajlova T, Stoinov S, et al. Fontolizumab (Huzaf), a humanized anti-IFN-gamma antibody, has clinical activity and excellent tolerability in moderate to severe Crohn's disease. *Gastroenterology* 2004; **127:** A332

44 Protein Design Labs reports progress on two humanized antibodies at International Organization of Inflammatory Bowel Disease. PDL Press release, March 2004. Available from: URL: http://www.pdl.com

45 **Van Assche G**, Dalle I, Noman M, Aerden I, Swijsen C, Asnong K, Maes B, Ceuppens J, Geboes K, Rutgeerts P. A pilot study on the use of the humanized anti-interleukin-2 receptor antibody daclizumab in active ulcerative colitis. *Am J Gastroenterol* 2003; **98**: 369-376

46 Protein Design Labs reports negative results in phase II clinical trial with daclizumab in ulcerative colitis. PDL Press release, May 2004. Available from: URL: http://www.pdl.com

47 **Creed TJ**, Norman MR, Probert CS, Harvey RF, Shaw IS, Smithson J, Anderson J, Moorghen M, Gupta J, Shepherd NA,

Dayan CM, Hearing SD. Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroid-resistant ulcerative colitis. *Aliment Pharmacol Ther* 2003; **18**: 65-75

48 **Creed T**, Probert C, Dayan C, Hearing S. Basiliximab (anti-CD25) for the treatment of steroid resistant ulcerative colitis. *Gastroenterology* 2004; **126**: A75

49 **Meenan J**, Spaans J, Grool TA, Pals ST, Tytgat GN, van Deventer SJ. Altered expression of alpha 4 beta 7, a gut homing integrin, by circulating and mucosal T cells in colonic mucosal inflammation. *Gut* 1997; **40**: 241-246

50 **Nakamura S**, Ohtani H, Watanabe Y, Fukushima K, Matsumoto T, Kitano A, Kobayashi K, Nagura H. In situ expression of the cell adhesion molecules in inflammatory bowel disease. Evidence of immunologic activation of vascular endothelial cells. *Lab Invest* 1993; **69:** 77-85

51 **Farstad IN**, Halstensen TS, Kvale D, Fausa O, Brandtzaeg P. Topographic distribution of homing receptors on B and T cells in human gut-associated lymphoid tissue: relation of L-selectin and integrin alpha 4 beta 7 to naive and memory phenotypes. *Am J Pathol* 1997; **150**: 187-199

52 **To SS**, Newman PM, Hyland VJ, Robinson BG, Schrieber L. Regulation of adhesion molecule expression by human synovial microvascular endothelial cells in vitro. *Arthritis Rheum* 1996; **39:** 467-477

53 **Gordon FH**, Lai CW, Hamilton MI, Allison MC, Srivastava ED, Fouweather MG, Donoghue S, Greenlees C, Subhani J, Amlot PL, Pounder RE. A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active Crohn's disease. *Gastroenterology* 2001; **121**: 268-274

54 **Ghosh S**, Goldin E, Gordon FH, Malchow HA, Rask-Madsen J, Rutgeerts P, Vyhnálek P, Zádorová Z, Palmer T, Donoghue S. Natalizumab for active Crohn's disease. *N Engl J Med* 2003; **348**: 24-32

55 **Rutgeerts P,** Colombel J, Enns R, Feagan B, Hanauer S, Lawrance I, Panaccione R, Sanders M, Schreiber S, Targan S, Van Deventer S, Sandborn W. Subanalyses from a phase 3 study on the evaluation of natalizumab in active Crohn's disease tharapy-1 (ENACT-1). *Gut* 2003; **52:** A239

56 **Sandborn W,** Colombel JF, Enns R, Feagan B, Hanauer S, Lawrance I, Panaccione R, Sanders M, Schreiber S, Targan S, Van Deventer S, Rutgeerts P. A phase III, double-blind, placebo-controlled study of the efficacy, safety, and tolerability of antegren (Natalizumab) in maintaining clinical response and remission in Crohn's disease (ENACT-2). *Gastroenterology* 2004; **127:** A332

57 **Gordon FH**, Hamilton MI, Donoghue S, Greenlees C, Palmer T, Rowley-Jones D, Dhillon AP, Amlot PL, Pounder RE. A pilot study of treatment of active ulcerative colitis with natalizumab, a humanized monoclonal antibody to alpha-4 integrin. *Aliment Pharmacol Ther* 2002; **16**: 699-705

58 **Miller DH**, Khan OA, Sheremata WA, Blumhardt LD, Rice GP, Libonati MA, Willmer-Hulme AJ, Dalton CM, Miszkiel KA, O'Connor PW. A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2003; **348**: 15-23

59 **Yednock TA**, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* 1992; **356**: 63-66

60 **Kleinschmidt-DeMasters BK**, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 2005; **353:** 369-374

61 **Langer-Gould A**, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med* 2005; **353:** 375-381

62 **Van Assche G,** Van Ranst M, Sciot R, Dubois B, Vermeire S, Noman M, Verbeeck J, Geboes K, Robberecht W, Rutgeerts P. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N Engl J Med* 2005; **353:** 362-368

63 **Feagan BG,** Greenberg G, Wild G, McDonald J, Fedorak R, Pare P, Kishimoto K, Gutierrez-Ramos JG, Krop J. Efficacy and safety of a humanized α4β7 antibody in active Crohn's disease (CD). *Gastroenterology* 2003; **124:** A25-26

64 **Feagan B,** Greenberg G, Wild G, McDonald J, Fedorak R, Pare P, Kishimoto K, Gutierrez-Ramos JG, Krop J, Vandervoort M. A randomized trial of a humanized α4β7 antibody in ulcerative colitis. *Gastroenterology* 2003; **125:** 606

65 **Bennett CF,** Condon TP, Grimm S, Chan H, Chiang MY. Inhibition of endothelial cell adhesion molecule expression with antisense oligonucleotides. *J Immunol* 1994; **152:** 3530-3540

66 **Yacyshyn BR**, Bowen-Yacyshyn MB, Jewell L, Tami JA, Bennett CF, Kisner DL, Shanahan WR Jr. A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 1998; **114**: 1133-1142

67 **Schreiber S**, Nikolaus S, Malchow H, Kruis W, Lochs H, Raedler A, Hahn EG, Krummenerl T, Steinmann G. Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. *Gastroenterology* 2001; **120**: 1339-1346

68 **Yacyshyn BR**, Chey WY, Goff J, Salzberg B, Baerg R, Buchman AL, Tami J, Yu R, Gibiansky E, Shanahan WR. Double blind, placebo controlled trial of the remission inducing and steroid sparing properties of an ICAM-1 antisense oligodeoxynucleotide, alicaforsen (ISIS 2302), in active steroid dependent Crohn's disease. *Gut* 2002; **51**: 30-36

69 **Yacyshyn BR**, Barish C, Goff J, Dalke D, Gaspari M, Yu R, Tami J, Dorr FA, Sewell KL. Dose ranging pharmacokinetic trial of high-dose alicaforsen (intercellular adhesion molecule-1 antisense oligodeoxynucleotide) (ISIS 2302) in active Crohn's disease. *Aliment Pharmacol Ther* 2002; **16**: 1761-1770

70 **van Deventer SJ**, Tami JA, Wedel MK. A randomised, controlled, double blind, escalating dose study of alicaforsen enema in active ulcerative colitis. *Gut* 2004; **53**: 1646-1651

71 **Miner P,** Wedel M, Bane B, Bradley J. An enema formulation of alicaforsen, an antisense inhibitor of intercellular adhesion molecule-1, in the treatment of chronic, unremitting pouchitis. *Aliment Pharmacol Ther* 2004; **19:** 281-286

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REVIEW

RNAi technology: A Revolutionary tool for the colorectal cancer therapeutics

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Abstract

With the many changes that have taken place in people's diet and lifestyle, colorectal cancer (CRC) has become a global concern. There were approximately 950 000 new cases diagnosed and 500 000 deaths recorded worldwide in 2000. It is the second most common type of cancer in the Western world, and it is the third most common type of digestive tumor in China. It is reported that the morbidity of CRC is 4.08/100 000 for men and 3.30/100 000 for women in China. Despite the rate of improvements in surgery, radiotherapy and chemotherapy, the overall five-year survival is around 50%. Therefore, novel treatment need to be developed in order to add to the therapeutic armamentarium. RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and causes degradation of mRNA homologous in sequence to the dsRNA.This new approach has been successfully adopted to inhibit virus replication and tumorigenicity. Recent reports have described DNA vector-based strategies for delivery of small interfering RNA (siRNA) into mammalian cells, further expanding the utility of RNAi. With the development of the RNAi technology and deeper understanding of this field, a promising new modality of treatment appeared, which can be used in combination with the existing therapies .We reviewed the proceedings on the actualities and advancement of RNAi technology for colorectal cancer therapeutics.

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Key words: RNAi; Colorectal cancer; Therapeutics

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INTRODUCTION

When viruses infect eukaryotic cells, or when transposons and transgenes are randomly integrated into host genomes, double-stranded RNA (dsRNA) is frequently produced from the foreign genes. Most eukaryotes, including humans, possess an innate cellular immune surveillance system that specifically responds to the presence of dsRNA and activates processes that act post-transcriptionally to silence the expression of the interloping genes[1-4]. This mechanism is now commonly referred to as RNA interference or RNAi[5]. During RNAi, long transcripts of dsRNA are rapidly processed into small interfering RNAs (siRNAs), which represent RNA duplexes of specific length and structure that finally guide sequence-specific degradation of mRNAs homologous in sequence to the siRNAs[6,7]. siRNAs may be the best tools for target validation in biomedical research today and cancer therapeutics, because of their exquisite specificity, efficiency and endurance of gene-specific silencing. Some researches about RNAi technology for colorectal cancer has been reported in recent years. However, these new approaches face serious problems before they can offer a useful adjuvant role for the clinical trials for patients with colorectal cancer.

MECHANISM OF dsRNA INTERFERENCE

The key enzyme required for the processing of long dsRNAs to siRNA duplexes is the RNase Ⅲ enzyme Dicer, which was characterized in extracts prepared from insect cells, C. elegans embryos, mouse cells and mast cells[8-10]. Dicer contains an N-terminal RNA helicase domain, a Piwi, Argonaute, Zwille/Pinhead (PAZ) domain[11], two RNase Ⅲ domains, and a C-terminal dsRNA-binding motif. The PAZ domain is also present in Argonaute proteins, whose genes represent a poorly characterized family present in dsRNA-responsive organisms. Argonaute1 (AGO1) and Argonaute2 (AGO2), two of the five Argonaute proteins of D. melanogaster, appear to be important for forming the mRNA-degrading sequence-specific endonuclease complex, also referred to as the RNA-induced silencing complex (RISC)[12,13]. Dicer and AGO2 appear to interact in D. melanogaster Schneider 2 (S2) cells, probably through their PAZ domains; however, RISC and Dicer activity are separable, and RISC is unable to process dsRNA to siRNAs, suggesting that Dicer is not

a component of RISC. Possibly, the interaction between Dicer and AGO2 facilitates the incorporation of siRNA into RISC[14]. The endonucleolytic subunit of RISC remains to be identified.

siRNA duplexes produced by the action of Dicer contain 5'-phosphates and free 3'-hydroxyl groups. The central base-paired region is flanked by two-to-three nucleotides of single-stranded 3'-overhangs. The 5' -phosphate termini of siRNAs is essential for guiding mRNA degradation[15]. Nevertheless, for their application in gene targeting experiments, siRNAs may be used without 5'-phosphate termini because a kinase activity in the cell rapidly phosphorylates the 5' ends of synthetic siRNA duplexes. Under certain circumstances (e.g., injection experiments in D. melanogaster), 5' -phosphorylated siRNA duplexes may have slightly enhanced properties as compared with 5'-hydroxyl siRNAs[16].

In C. elegans, introduction of approximately 300 bp dsRNA corresponding to a segment of the targeted gene may also give rise to the phenomenon of transitive RNAi[17]. Transitive RNAi is characterized by the spread of silencing outside of the region targeted by the initiator dsRNA. Presumably, targeted mRNA serves as template for RNA-dependent RNA polymerase (RdRP) and forms new dsRNA that is processed by Dicer. Thus, secondary siRNAs are generated which may cleave the mRNA out of the region targeted by the ancestral dsRNA.

Until recently, the application of siRNAs in somatic cells was restricted to the delivery of chemically or enzymatically synthesized siRNAs[18-20], but methods for intracellular expression of small RNA molecules have now been developed. Endogenous delivery is possible by inserting DNA templates for siRNAs into RNA polymerase Ⅲ (pol Ⅲ) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6 or the human RNase P RNA H1. Two approaches are available for expressing siRNAs: (1) The sense and antisense strands constituting the siRNA duplex are transcribed from individual promoters[20-22], or (2) siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs after intracellular processing by Dicer[23,24].The transfection of cells with plasmids that encode siRNAs, therefore, represents an alternative to direct siRNA transfection. The insertion of siRNA expression cassettes into (retro)viral vectors will also enable the targeting of primary cells refractory to transfection or electroporation of plasmid DNA.

siRNAs AS NOVEL THERAPEUTIC PLATFORM TECHNOLOGY

VEGF is one of the archetypal angiogenic growth factors and has received considerable attention. VEGF is a homodimeric 45 kDa glycoprotein, 5 different isoforms of which are reportedly expressed by endothelial cells. VEGF specifically acts on endothelial cells binding to a growing number of endothelial tyrosine kinase receptors including VEGFR-1 and VEGFR-2. Inhibition of VEGF activity or disabling the function of its receptors has been shown to inhibit both tumor growth and metastasis in a variety of animal tumor models[25,26]. Given the different isoforms and their various functions, the development of this RNAi technology and its ability to target specific VEGFs should facilitate both a greater understanding of this field and also the development of improved therapeutics

Single base pair mutations that alter the function of tumor suppressor genes and oncogenes occur frequently during oncogenesis. The guardian of the genome, p53, is inactivated by point mutation in more than 45%-60% of human colorectal cancers. Synthetic small inhibiting RNAs (siRNAs) are highly sequence-specific reagents and discriminate between single mismatched target RNA sequences, and may represent a new avenue for gene therapy. Martinez LA[27] demonstrated that a single base difference in siRNAs discriminates between mutant and WT p53 in cells expressing both forms, resulting in the restoration of WT protein function. Therefore, siRNAs may be used to suppress expression of point-mutated genes and provide the basis for selective and personalized antitumor therapy.

The products of bcl-2 genes are involved in the regulation of apoptosis and proliferation and are associated with prognosis in several malignancies, including colorectal adenocarcinoma. A statistically significant inverse association was found between Bcl-2 score and tumor recurrence. It is reported that some researchers use mRNA-cDNA interference for silencing bcl-2 expression in human LNCaP cells[28]. These findings indicate that a novel gene silencing system may play a useful adjuvant role in the majority of patients with colorectal cancer.

One of the major limitations of current chemotherapy regimes is the bone marrow toxicity associated with these drugs. However, it is well recognized that subpopulations of tumor cells are resistant to particular chemotherapeutic agents and continue to grow in a selective manner in the presence of such drugs. These cells contain specific genes which render them resistant to particular compounds. One such gene is the multiple drug resistance (MDR1) gene which confers resistance to vinca alkaloids (vinblastine, vincristine), anthracyclins (adriamycin, daunorubicin), etoposide and paclitaxel. For reversal of MDR1 gene-dependent multidrug resistance (MDR), two small interfering RNA (siRNA) constructs were designed to inhibit MDR1 expression by RNA interference. Some data indicate that this approach may be applicable to cancer patients as a specific means to reverse tumors with a P-glycoprotein-dependent MDR phenotype back to a drug-sensitive one[29].

Telomerase is an attractive molecular target toward which to direct cancer therapeutic agents because telomerase activity is present in most malignant cells but undetectable in most normal somatic cells. Kosciolek BA, *et al*[30] evaluated the ability of siRNA to inhibit telomerase activity in human cancer cells. In their research, human cancer cell lines were transfected with 21 nt double-stranded RNA homologous to either the catalytic subunit of telomerase (human telomerase reverse transcriptase) or its template RNA [human telomerase RNA(hTR)]. Both types of agents reduced telomerase activity in a variety of human cancer cell lines representing both carcinomas and sarcomas. Inhibition was dose-dependent. Telomerase

inhibition by siRNA is notable because telomerase is regarded as restricted to the nucleus, whereas RNA interference is commonly regarded as restricted to the cytoplasm. Their results showed that telomerase activity in human cancer cells can be inhibited by short dsRNAs (siRNAs) targeting telomerase components. Inhibition was shown in a variety of carcinoma cell lines (HCT-15 colon carcinoma, HeLa cervical carcinoma, NCI H23 lung carcinoma, and A431 epidermoid carcinoma).

CRC typically develops over decades and involves multiple genetic events. This has led to the development of a multistep model of colorectal tumorigenesis In order to identify genes that are important in the development of CRC, RNAi was used to disrupt expression of two of the genes identified by microarray analysis in a colon tumor cell line, HCT116. HCT116 cells were derived from a human colon carcinoma, and showed mutations in β-catenin and K-ras, but possessed wild-type p53[31]. By examining the growth characteristics of these cells after RNAi both in vivo and in vitro, they hoped to identify targets critical for growth, apoptosis, and/or metastasis. It is clear from these results that siRNA directed against c-myc and survivin lowers the levels of these proteins without affecting the levels of a control protein, β-tubulin. Furthermore, transfection of siRNA oligonucleotides directed against both genes was as effective in reducing protein expression, as were experiments targeting each gene separately. In summary, they have demonstrated that the use of RNAi when coupled with microarray analysis provides an excellent system to define the role of specific genes that are dysregulated in cancer on both the *in vitro* and in vivo growth of the tumor[32].

Somatic changes in CpG dinucleotide methylation occur quite commonly in human cancer cell DNA. Relative to DNA from normal human colonic cells, DNA from human colorectal cancer cells typically displays regional CpG dinucleotide hypermethylation amid global CpG dinucleotide hypomethylation. The role of the maintenance DNA methyltransferase (DNMT1) in the acquisition of such abnormal CpG dinucleotide methylation changes in colorectal cancer cells remains obvious. Some research indicated that human colorectal carcinogenesis is accompanied by a progressive dysregulation of DNMT1 expression and suggest that abnormalities in DNMT1 expression may contribute to the abnormal CpG dinucleotide methylation changes characteristic of human colorectal carcinoma cell DNA[33]. Recently, there is a report that the controlled knockdown of DNA methyltransferase 1 (DNMT1) in human cancer resulted in growth arrest[34]. Thus the method allows for a highly controlled approach to gene knockdown.

CONCLUSION

Since biotechnologists have adopted RNAi, it has already earned a place among the major technology platforms. Despite promising data, there are several challenges that need to be faced before RNAi can be used in patients. These include mode of delivery, the precise sequence of the siRNA or shRNA used, and cell type specificity. There are possible toxicities related to silencing of partially homologous genes or induction of global gene suppression by activating the interferon response. Another potential problem is the inhibition of the function of endogenous miRNAs through competition for the RNAi machinery. Despite these hurdles, RNAi provides the opportunity to pursue an exciting new therapeutic approach to treat colorectal cancer[35].

REFERENCES

1 **Hammond SM**, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001; **2**: 110-119

2 **Hutvágner G**, Zamore PD. RNAi: nature abhors a double-strand. *Curr Opin Genet Dev* 2002; **12**: 225-232

3 **Bergstrom CT**, Antia R. On RNA interference as template immunity. *J Biosci* 2005; **30**: 295-297

4 **Waterhouse PM**, Wang MB, Lough T. Gene silencing as an adaptive defence against viruses. *Nature* 2001; **411**: 834-842

5 **Fire A**, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998; **391**: 806-811

6 **Elbashir SM**, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 2001; **15**: 188-200

7 **Elbashir SM**, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J* 2001; **20**: 6877-6888

8 **Bernstein E**, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001; **409**: 363-366

9 **Billy E**, Brondani V, Zhang H, Müller U, Filipowicz W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci USA* 2001; **98**: 14428-14433

10 **Yin D**, Stuart CA. Gene silencing using small interference RNA in mast cells. *Methods Mol Biol* 2006; **315**: 333-339

11 **Cerutti L**, Mian N, Bateman A. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem Sci* 2000; **25**: 481-482

12 **Hammond SM**, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 2000; **404**: 293-296

13 **Williams RW**, Rubin GM. ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. *Proc Natl Acad Sci USA* 2002; **99**: 6889-6894

14 **Hammond SM**, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 2001; **293**: 1146-1150

15 **Nykänen A**, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 2001; **107**: 309-321

16 **Boutla A**, Delidakis C, Livadaras I, Tsagris M, Tabler M. Short 5'-phosphorylated double-stranded RNAs induce RNA interference in Drosophila. *Curr Biol* 2001; **11**: 1776-1780

17 **Sijen T**, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 2001; **107**: 465-476

18 **Donzé O**, Picard D. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res* 2002; **30**: e46

19 **Paddison PJ**, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002; **16**: 948-958

20 **Yu JY**, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 2002; **99**: 6047-6052

21 **Lee NS**, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra

P, Rossi J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 2002; **20**: 500-505

22 **Miyagishi M**, Taira K. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 2002; **20**: 497-500

23 **Paul CP**, Good PD, Winer I, Engelke DR. Effective expression of small interfering RNA in human cells. *Nat Biotechnol* 2002; **20**: 505-508

24 **Sui G**, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* 2002; **99**: 5515-5520

25 **Harper SQ**, Davidson BL. Plasmid-based RNA interference: construction of small-hairpin RNA expression vectors. *Methods Mol Biol* 2005; **309**: 219-235

26 **Holash J**, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci USA* 2002; **99**: 11393-11398

27 **Martinez LA**, Naguibneva I, Lehrmann H, Vervisch A, Tchénio T, Lozano G, Harel-Bellan A. Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. *Proc Natl Acad Sci USA* 2002; **99**: 14849-14854

28 **Lin SL**, Chuong CM, Ying SY. A Novel mRNA-cDNA interference phenomenon for silencing bcl-2 expression in human LNCaP cells. *Biochem Biophys Res Commun* 2001; **281**: 639-644

29 **Nieth C**, Priebsch A, Stege A, Lage H. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). *FEBS Lett* 2003; **545**: 144-150

30 **Kosciolek BA**, Kalantidis K, Tabler M, Rowley PT. Inhibition of telomerase activity in human cancer cells by RNA interference. *Mol Cancer Ther* 2003; **2**: 209-216

31 **Lu X**, Errington J, Curtin NJ, Lunec J, Newell DR. The impact of p53 status on cellular sensitivity to antifolate drugs. *Clin Cancer Res* 2001; **7**: 2114-2123

32 **Williams NS**, Gaynor RB, Scoggin S, Verma U, Gokaslan T, Simmang C, Fleming J, Tavana D, Frenkel E, Becerra C. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clin Cancer Res* 2003; **9**: 931-946

33 **De Marzo AM**, Marchi VL, Yang ES, Veeraswamy R, Lin X, Nelson WG. Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res* 1999; **59**: 3855-3860

34 **Matsukura S**, Jones PA, Takai D. Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res* 2003; **31**: e77

35 **Sakamoto KM**. Knocking down human disease: potential uses of RNA interference in research and gene therapy. *Pediatr Res* 2004; **55**: 912-913

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GASTRIC CANCER

Suppression of bile acid synthesis by thyroid hormone in primary human hepatocytes

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Abstract

AIM: It is known that thyroid hormones alter the bile acid metabolism in humans, however the effect on individual enzymes has been difficult to elucidate. This is mainly due to the lack of human liver cell lines producing bile acids. We used cultures of primary human hepatocytes to study the effects of triiodothyronine (T_3) on bile acid synthesis.

METHODS: Primary hepatocytes were isolated from liver tissue obtained from three different patients undergoing liver resection due to underlying malignancy. The hepatocytes were cultured under serum-free conditions and treated from d 1 to d 5 with culture containing 0.1 - 1000 nmol/L of T_3. Bile acid formation and mRNA levels of key enzymes were analysed.

RESULTS: The lowest concentration of T_3 decreased cholic acid (CA) formation to 43%-53% of controls and chenodeoxycholic acid (CDCA) to 52%-75% of controls on d 5. The highest dose further decreased CA formation to 16%-48% of controls while CDCA formation remained at 50%-117% of controls. Expression of mRNA levels of cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1) dose-dependently decreased. Sterol 27-hydroxylase (CYP27A1) levels also decreased, but not to the same extent.

CONCLUSION: T_3 dose-dependently decreased total bile acid formation in parallel with decreased expression of CYP7A1 and CYP8B1. CA formation is inhibited to a higher degree than CDCA, resulting in a marked decrease in the CA /CDCA ratio.

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Key words: Hepatocytes; Human; Bile acids; CYP7A1; CYP8B1; CYP27A1

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INTRODUCTION

The effect of thyroid hormone on human bile acid synthesis is still unclear. Several *in vivo* observations have suggested that thyroid hormone influences the bile acid synthesis[1-3]. However, due to the lack of human *in vitro* tools such as cell lines synthesizing normal bile acids, investigators have not been able to study this at a cellular level. We have previously shown that primary human hepatocytes cultured under serum-free conditions in the presence of matrixgel (EHS) synthesize and conjugate normal bile acids[4,5]. We also showed that under these conditions they synthesize bile acids with the same capacity and composition as under similar *in vivo* conditions.

In the conversion of cholesterol into bile acids two main pathways are recognized[6-8]. The neutral pathway is initiated by the 7α-hydroxylation of cholesterol, catalyzed by the microsomal rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1). The acidic pathway is initiated by the 27-hydroxylation of cholesterol, catalyzed by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1). In the acidic pathway the microsomal oxysterol 7α-hydroxylase (CYP7B1) mediates the 7α-hydroxylation. Humans synthesize two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA). CA has a hydroxyl group at the 12th carbon distinguishing it from CDCA. The hydroxyl group is introduced by the microsomal enzyme sterol 12α-hydroxylase (CYP8B1). Therefore, the total amount of intermediates (from either pathway) that undergoes 12α-hydroxylation determines the ratio of CA to CDCA. Both pathways can synthesize CA and CDCA, however the acidic pathway is believed to produce mainly CDCA. The most important mechanism for regulation of bile acid synthesis is the portal flux of bile acids returning to the liver. The loss of bile acids in the intestine (5%-10%) is compensated by *de novo* synthesis.

In this study we examined the effect of different doses

of triiodothyronine (T_3) on bile acid formation and on mRNA levels of *CYP7A1*, *CYP8B1* and *CYP27A1* in cultures of primary human hepatocytes.

MATERIALS AND METHODS

Materials

RPA Ⅲ™ kit and pTRI-cyclophilin-Human antisense control template were purchased from Ambion Inc.(Huntingdon, UK). ^{35}S-UTP was purchased from Hartmann Analytic GmbH(Braunschweig, Germany). William's medium E with glutamax, gentamycin, penicillin-streptomycin, Dulbecco's phosphate buffered saline, Trizol reagent and Superscript Ⅱ RT were purchased from Invitrogen (Täby, Sweden). Actrapid (insulin) 100 IU/mL was from Novo Nordisk Pharma(Malmö, Sweden). Collagenase XI-S (Cat. No. C7557), methylthaizolyldiphenyl-tetrazolium bromide (Cat. No. M5655) and triiodothyronine (Cat. No. T6397) were purchased from Sigma-Aldrich (Sweden). Random hexamers (Cat. No. C1181), recombinant RNasin ribonuclease inhibitor (Cat. No. N2511) and RQ DNase I (Cat. No. M6101) were purchased from Promega (Southampton, UK). Taqman universal master mix (Cat. No. 4304437) and human cyclophilin pre-developed assay (now discontinued and replaced by Human PPIA endogenous control (Cat. No. 4310883E) were purchased from Applied Biosystems (Stockholm, Sweden). Dithiothreitol (Cat. No. 708984) was purchased from Roche(Bromma, Sweden).

Isolation of primary human hepatocytes

Human liver tissue was obtained from three different patients undergoing reduction hepatectomy for metastatic cancer to the liver where normal liver tissue was resected due to the localization of the tumor. Patient A was a 62 year old female, patient B a 47 year old male and patient C a 49 year old female.

Approval to use parts of resected human liver specimens for research was given by the ethics committee at Huddinge University Hospital and the patient's informed consent was obtained.

Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as described in detail by Li *et al*[9]. In brief, liver tissue weighing between 20-150 g was cannulated in 1-2 vessels depending on the size and appearance. The tissue was then perfused with EGTA buffer for about 15 min at a flow rate of 20 mL/min, followed by perfusion with CO_2 (5%) buffered solution containing collagenase XI-S (200 mg /L) at 40℃ for approximately 75 min.

Culture conditions

Hepatocytes (3.5×10^6) were cultured under standard conditions in 60 mm culture dishes containing 200 μL EHS matrigel and 3 mL William's E medium with glutamax, supplemented with insulin (2 IU/L), penicillin G sodium (100 U/mL), streptomycin sulphate (100 μg/mL) and gentamycin (85 μg/mL). Cell culture media were collected and renewed everyday. Three 60 millimeter culture dishes each containing 3.5×10^6 hepatocytes were used for each treatment. The cells and media from three dishes were pooled when harvested. The cells were subjected to different concentrations of T_3 (0.1, 1, 10, 100 and 1000 nmol/L) during four days, from d 1 to d 5. On d 5 cell culture media were collected for bile acid analysis and the cells were harvested for RNA isolation and subsequent quantification of specific mRNAs.

Analysis of bile acids in cell culture media

Bile acids in cell culture media were analysed as previously described[4]. Briefly, 2 mL of the harvested culture medium, together with deuterium labelled cholic acid (D_5) and chenodeoxycholic acid (D_4), was subjected to basic hydrolyzation to remove conjugates and the bile acids were extracted using acidic ether. Bile acids were analysed by gas chromatography-mass spectrometry, as described previously[1,10].

Isolation of total RNA

Total RNAs were isolated using Trizol reagent as described by the manufacturer. RNA concentration was determined by spectrophotometry at 260 nm. The purity of the RNA was determined by spectrophotometry at 280 nm and the integrity checked by agarose gel electrophoresis stained with ethidium bromide.

Quantification of CYP7A1 by real time PCR

mRNA levels of CYP7A1 were determined using quantitative single-plex real time PCR. From each group, 40 μg of total RNA was incubated with 5 U of RQ DNase, 5 μL 10 × RQ buffer in a total volume of 50 μL at 37℃ for 20 min, at 20℃ for 15 min and finally at 70℃ for 10 min. From this, 4 μg of DNase treated RNA was used for cDNA synthesis by mixing together with 2 μL random hexamer primers (100 ng/μL), 1 μL dNTP (10 mmol/L of each) in a total volume of 12 μL. After incubation at 65℃ for 5 min, 25℃ for 10 min and 42℃ for 2 min, 2 μL of DTT (0.1 mol/L), 1 μL of RNasin (40 U/μL), 4 μL 5 × first strand buffer and 1 μL superscript II (200 U/μL) were added. The incubation was continued at 42℃ for 50 min and at 70℃ for 10 min. For real time PCR 3 μL undiluted cDNA/sample was used in triplicates.

Sequences for CYP7A1 primers and probe were kindly provided by Dr. Hermansson, AstraZeneca, Mölndal, Sweden. Reversed primer is: 5'AGA GCA CAG CCC AGG TAT G 3'; forward primer, 5' TGA TTT GGG GGA TTG CTA TA 3' (300 mmol/L); and probe 5' TGG TTC ACC CGT TTG CCT TCT CCT 3' (200 mmol/L). The taqman probe was labelled with FAM and TAMRA. The assays were performed according to ABI user bulletin #2 protocol in a total volume of 25 μL. VIC labelled human cyclophilin pre-developed taqman assay from Applied Biosystems was used for internal control. The assay was performed and analysed using ABI Prism 7700 sequence detector system.

Quantification of CYP8B1 and CYP27A1 by RNase protection assay

Abundance of specific mRNA was quantified using RPA III™. cRNA probes were synthesized using T3- or T7-polymerase and labelled with ^{35}S-UTP. Cyclophilin was

used as an internal standard and gave a protected fragment size of 103 bp.

The protected fragment size of the probe for CYP27A1 was 297 bp and for CYP8B1 442 bp. The assay was performed according to the manufacturer's protocol. In brief, 10-15 μg of total RNA was co-precipitated in 2.5 volumes of ethanol and 0.5 mol/L NH_4OAc with about 80 000 cpm of each probe at -20℃ for 15 min. Following centrifugation at 15 000 rpm for 15 min the pellets were dissolved in 10 μL hybridisation buffer, heated to 95℃ for 3 min and incubated at 46℃ overnight. The hybridised RNA samples were then treated with RNase A and RNase A/T1 diluted 1:50 in RNase solution at 37℃ and inactivated after 30 min by adding inactivation/precipitation solution. Following precipitation at -20℃ for 15 min the samples were centrifuged at 15 000 r/min for 15 min and the pellets were air dried and dissolved in 10 μL of loading buffer.

The protected fragments were separated on a denaturing 5% polyacrylamide urea gel and detected and quantified using a Fuji BAS 1800 Phospho-imager.

Statistical analysis

The significance of differences between groups for the mRNA analysis was tested by a one-way ANOVA followed by a post-hoc test. To stabilize the variances, data were logarithmically transformed. Due to variances between patients, statistical analysis of the bile acid formation could not be performed.

RESULTS

Purity of hepatocyte preparations

We have analyzed the purity of the preparation of hepatocytes that we plated. Following the final low speed centrifugation step the cell pellet was resuspended in plating media and samples of the cell preparation were spotted onto microscopic glass slides, air dried and fixed in formalin. Preparations from 2 separate human cases were analyzed. Duplicate slides were exposed to antibodies for low molecular weight cytokeratins (AE1/AE3), albumin or hepatocyte specific antibody HEPR. The results showed that 93% of the cells in the preparation reacted with antibodies to cytokeratin, albumin and HEPR, indicating that 93% of the cells plated on culture dishes were epithelial (cytokeratin positive) and parenchymal hepatocytes (HEPR and albumin positive). Most of the remaining cells were small and appeared to be hematopoietic. Hematopoietic cells would not be expected to attach to culture dishes and would be removed by media changes.

Patient A

The cells were treated for 4 d (from d 1 to d 5) with different concentrations of T_3. The cell culture media were changed daily and collected on d 5 of culture after 24 h incubation. In control cells the total bile acid formation was 1515 ng/mL of culture media (920 ng CA and 595 ng CDCA per mL) and the CA/CDCA ratio 1.5. Treatment with 0.1 nmol/L of T_3 decreased total formation by 53% to 710 ng/mL and lowered the CA/CDCA ratio to 1.3 (Table 1). Higher doses of T_3 decreased bile acid formation even further in a dose dependent manner, down to 36% of control with a CA/CDCA ratio of 0.8 for the highest dose, 1000 nmol/L.

Table 1 Formation of CA and CDCA in primary human hepatocytes obtained from 3 patients

Patient	Addition of T_3	CA ng/mL	CDCA Medium/24 h	Ratio CA/CDCA
A	Control	920	595	1.5
	0.1 nmol/L	400	310	1.3
	1 nmol/L	375	375	1.0
	10 nmol/L	340	390	0.9
	100 nmol/L	250	285	0.9
	1 μmol/L	245	295	0.8
B	Control	280	60	4.7
	0.1 nmol/L	135	45	3.0
	1 nmol/L	95	70	1.4
	10 nmol/L	55	60	0.9
	100 nmol/L	45	65	0.7
	1 μmol/L	45	70	0.6
C	Control	805	502	1.6
	0.1 nmol/L	430	345	1.2
	1 nmol/L	477	392	1.2
	10 nmol/L	395	425	0.9
	100 nmol/L	372	405	0.9
	1 μmol/L	382	420	0.9

The cells were cultured in a serum-free medium and treated with different concentrations of T_3 for 5 d. Bile acids were measured in the cell-free medium. CA: Cholic acid; CDCA: Chenodeoxycholic acid.

Expression of CYP7A1 mRNA levels was decreased by all doses of T_3 tested. Addition of 0.1 nmol/L of T_3 decreased CYP7A1 mRNA levels from 4.45 to 0.54 (arbituary value) (12% of control), 10 nmol/L to 0.44 and 1000 nmol/L further down to 0.34 (8% of control). Two of the measure points were lost due to human error (Figure 1).

Similarly, expression of CYP8B1 mRNA decreased with T_3 treatment. Addition of 0.1 nmol/L of T_3 decreased CYP8B1 from 0.226 to 0.056 (arbituary value) (12% of control), 10 nmol/L to 0.028 and 1000 nmol/L to 0.017 (8% of control) (Figure 1).

Also CYP27A1 mRNA levels decreased with T_3 treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 1.050 to 0.408 (arbituary value) (39% of control), 10 nmol/L to 0.373 and 1000 nmol/L to 0.334 (32% of control) (Figure 1).

Patient B

The bile acid formation in hepatocytes from patient B was much lower than in cells from patients A and C. In control cells the total bile acid formation was 340 ng/mL of culture media (280 ng CA and 60 ng CDCA per mL) and the CA/CDCA ratio was 4.7. Treatment with 0.1 nmol/L of T_3 decreased total formation by 47% to 180 ng/mL, and lowered CA/CDCA ratio to 3.0 (Table 1). Higher doses of T_3 decreased bile acid formation even further down to 34% of control and CA/CDCA ratio to 0.6 for

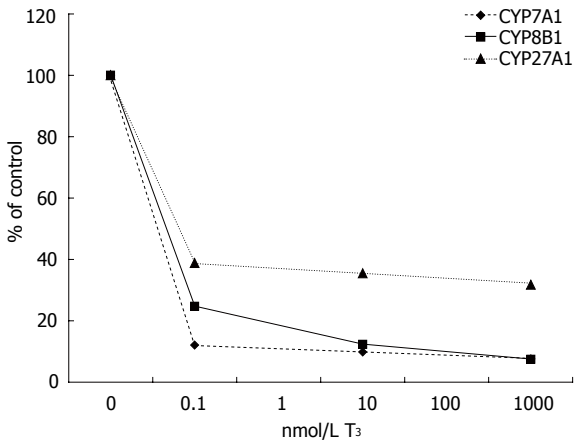

Figure 1 Patient A, effect of T_3 on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

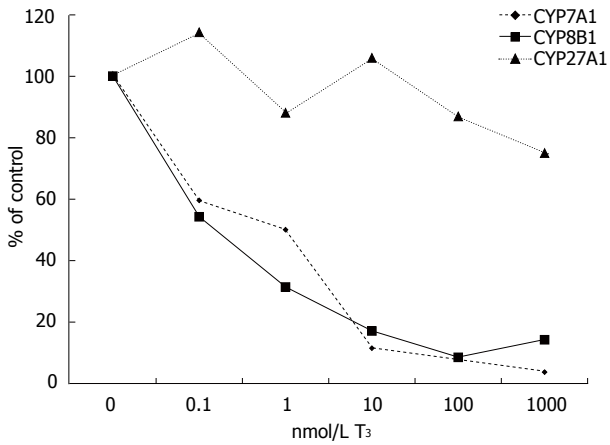

Figure 2 Patient B, effect of T_3 on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T_3 tested but not to the same extent as in cells from patient A. Addition of 0.1 nmol/L of T_3 decreased CYP7A1 mRNA levels from 0.745 to 0.444 (arbituary value) (60% of control), 1 nmol/L to 0.373, 10 nmol/L to 0.086, 100 nmol/L to 0.063 and 1000 nmol/L further down to 0.028 (4% of control) (Figure 2).

Similarly, expression of CYP8B1 decreased with T_3 treatment. Addition of 0.1 nmol/L of T_3 decreased CYP8B1 mRNA levels from 0.175 to 0.095 (arbituary value) (54% of control), 1 nmol/L to 0.055, 10 nmol/L to 0.030, 100 nmol/L to 0.015 and 1000 nmol/L further down to 0.025 (14% of control) (Figure 2).

T_3 had a modest effect on the expression of CYP27A1 in patient B. Addition of 0.1 nmol/L of T_3 changed CYP27A1 mRNA levels from 0.420 to 0.480 (arbituary value) (114% of control), 1 nmol/L to 0.370, 10 nmol/L to 0.445, 100 nmol/L to 0.365 and 1000 nmol/L down to 0.315 (75% of control) (Figure 2).

Patient C

In control cells total bile acid formation was 1308 ng/mL of culture media (805 ng CA and 502 ng CDCA per mL) and the CA/CDCA ratio was 1.6. Treatment with 0.1 nmol/L of T_3 decreased the total formation by 41% to 775 ng/mL and lowered the CA/CDCA ratio to 1.2 (Table 1). Higher doses of T_3 decreased bile acid formation to 61% of control and lowered the CA/CDCA further down to 0.9 for the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T_3 tested, but not as strongly as in patients A and B. Addition of 0.1 nmol/L of T_3 decreased CYP7A1 mRNA levels from 3.15 to 1.64 (arbituary value) (52% of control), 1 nmol/L to 2.06, 10 nmol/L to 1.06, 100 nmol/L to 0.81 and 1000 nmol/L further down to 0.65 (21% of control) (Figure 3).

Similarly, expression of CYP8B1 mRNA decreased with T_3 treatment. Addition of 0.1 nmol/L of T_3 decreased CYP8B1 mRNA levels from 0.093 to 0.070 (arbituary value) (75% of control), 1 nmol/L to 0.040, 10 nmol/L to 0.050, 100 nmol/L and 1000 nmol/L both to

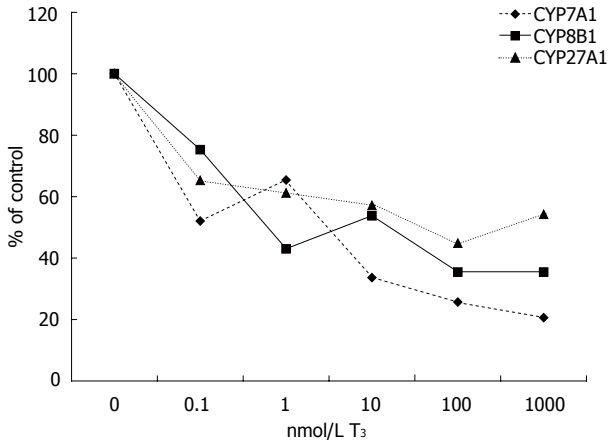

Figure 3 Patient C, effect of T_3 on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

0.033 (35% of control) (Figure 3).

Also CYP27A1 mRNA levels decreased with T_3 treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 0.245 to 0.160 (arbituary value) (65% of control), 1 nmol/L to 0.150, 10 nmol/L to 0.140, 100 nmol/L to 0.110 and 1000 nmol/L to 0.133 (54% of control) (Figure 3).

Following logarithmical transformation, statistical analysis of mRNA levels from all three patients showed that the highest dose (1000 nmol/L) of T_3 significantly decreased CYP7A1 mRNA levels ($P < 0.05$). CYP8B1 mRNA levels was significantly decreased by 10 and 100 nmol/L of T_3. Treatment with T_3 did not significantly decrease CYP27A1 mRNA levels.

DISCUSSION

In accordance with our previous studies[4,5,11,12], CA and CDCA, constituted more than 95% of total sterols and the ratio between the two bile acids averaged 2.6.

T_3 dose-dependently decreased formation of bile acids to 44% of controls. The reduction of CA formation was greater than the decline in CDCA formation and

resulted in a marked decrease in the CA/CDCA ratio. These results are in reasonable agreement with previous *in vivo* studies. Angelin *et al*[13] studied bile acid metabolism in hypothyroid patients before and during replacement therapy to euthyroid state and found that CDCA synthesis was stimulated by approximately 40%, whereas CA did not change with hormone treatment. However, the relative concentration of deoxycholic acid (DCA) in bile decreased from 30% to 19% and CDCA concomitantly increased with hormone therapy. This was confirmed by Kosuge *et al*[2] who concluded that the characteristic effect of thyroid hormone on serum bile acid composition in man is the shift from the "family" of cholic to that of CDCA by showing that DCA is the most prominent bile acid in hypothyroid patients and that CDCA is the most prominent bile acid in hyperthyroid patients. Paulezki *et al*[3] studied bile acid metabolism in hyperthyroid patients before and during treatment to euthyroid state and showed that thyroid hormone excess caused a 34% reduction in CA synthesis with no apparent change of CDCA synthesis and a 20% decrease in total bile acid synthesis. The authors suggested that thyroid hormone inhibited hepatic 12α-hydroxylation.

In contrast to this, Sauter *et al* failed to show any effect of T_3 treatment on the serum levels of the bile acid intermediate 7α-hydroxy-4-cholestene-3-one (C4) (believed to reflect CYP7A1 activity *in vivo*) in hypo- and hyperthyroidism before and after treatment to euthyroid state[14]. A recent study by Galman *et al* showed that there are marked diurnal changes of C4 levels during the day[15]. The authors could also see a weak but significant correlation of C4 with cholesterol and therefore corrected C4 levels for cholesterol. These findings make it difficult to evaluate the results of the C4 levels of the mentioned study. Also, the fact that C4 is the main substrate for CYP8B1 and that one study suggested that CYP8B1 might be inhibited by thyroid hormone needs to be considered.

The mRNA levels of the rate-limiting enzyme CYP7A1 in the neutral pathway were also dose-dependently decreased by T_3. Our data support the human promoter studies by Wang *et al* who found that thyroid hormone repressed CYP7A1 activity[16]. Furthermore, Drover *et al* found a distinct thyroid hormone response element that mediates T_3 repression in the human CYP7A1 promoter[17]. This is in contrast to the study in rats where Crestani *et al* failed to demonstrate an effect of thyroid hormone on the *Cyp7a1* promoter activity[18]. However addition of 1 μmol/L of T_4 to primary rat hepatocytes increased the transcriptional activity of *Cyp7a1* by 3.7 fold but did not alter steady state mRNA levels[19].

In the present study we did not measure the enzyme activity of CYP7A1 because of lack of material, but since the effects on the mRNA levels were in agreement with the effects on the bile acid formation we believed that it would be unlikely that T_3 would have an opposite effect on the enzyme activity.

Similar to CYP7A1, the CYP8B1 mRNA levels were also dose-dependently decreased by T_3. Although previous *in vivo* data pointed towards an effect on CYP8B1, this is to our knowledge the first report of the effects of T_3 on human CYP8B1 mRNA levels. In rats however, T_3 has been shown to suppress both Cyp8b1 activity and mRNA levels[20].

Treatment with T_3 also decreased CYP27A1 levels, but to a much lower degree than CYP7A1 and CYP8B1. This finding is in line with the contention that CYP27A1 is regulated to a lower degree than CYP7A1 in humans, as has been shown in connection with bile acid mediated feedback regulation[21]. According to a previous study by Stravitz *et al*[22] *Cyp27a1* is not regulated by thyroid hormone at all in rat.

In a previous study[4] we investigated the effect of the combination of T_3 and dexamethasone on bile acid synthesis in rat *vs* human hepatocytes. Since it was previously shown that T_3 and dexamethasone had a synergistic effect on *Cyp7a1* mRNA levels in primary rat hepatocytes[19] and since neither T_3 nor dexamethasone alone had any effect in rat hepatocytes, we did not focus on the effect of T_3 or dexamethasone alone in primary human hepatocytes. However, we could see that T_3 and dexamethasone alone tended to decrease bile acid formation but had no consistent effect on CYP7A1 mRNA levels (data not shown). The results of the present study and the fact that combination of T_3 and dexamethasone did not change human bile acid formation or CYP7A1 mRNA levels in our prior study, suggest that dexamethasone might have an effect on human bile acid synthesis. This is very interesting and needs to be elucidated in future studies.

The concentrations of T_3 used in this study ranged from 0.1 nmol/L to 1000 nmol/L. The highest dose was included because this is a dose commonly used in combination with dexamethasone in cultures of primary rat hepatocytes. Reduction of methylthaizolyldiphenyl-terazolium bromide (MTT) showed no signs of cytotoxicity in the dose range used here (data not shown). It should be emphasized that the most dramatic effects were seen with the lowest T_3 concentration, supporting that the effects observed are physiological.

In conclusion, it has been shown that T_3 dose-dependently decreases bile acid formation and expression levels of CYP7A1 and CYP8B1 mRNAs in primary human hepatocytes. CYP27A1 is also decreased, but to a lower degree. CA formation is inhibited to a higher degree than CDCA formation, resulting in a marked decrease in the CA/CDCA ratio.

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REFERENCES

1 **Angelin B**, Bjökhem I, Einarsson K. Individual serum bile acid concentrations in normo- and hyperlipoproteinemia as determined by mass fragmentography: relation to bile acid pool size. *J Lipid Res* 1978; **19**: 527-537

2 **Kosuge T**, Beppu T, Kodama T, Hidai K, Idezuki Y. Serum

bile acid profile in thyroid dysfunction and effect of medical treatment. *Clin Sci* (Lond) 1987; **73**: 425-429
3 **Pauletzki J**, Stellaard F, Paumgartner G. Bile acid metabolism in human hyperthyroidism. *Hepatology* 1989; **9**: 852-855
4 **Ellis E**, Goodwin B, Abrahamsson A, Liddle C, Mode A, Rudling M, Bjorkhem I, Einarsson C. Bile acid synthesis in primary cultures of rat and human hepatocytes. *Hepatology* 1998; **27**: 615-620
5 **Axelson M**, Ellis E, Mörk B, Garmark K, Abrahamsson A, Björkhem I, Ericzon BG, Einarsson C. Bile acid synthesis in cultured human hepatocytes: support for an alternative biosynthetic pathway to cholic acid. *Hepatology* 2000; **31**: 1305-1312
6 **Russell DW**. Cholesterol biosynthesis and metabolism. *Cardiovasc Drugs Ther* 1992; **6**: 103-110
7 **Princen HM**, Post SM, Twisk J. Regulation of Bile Acid Biosynthesis. *Current Pharmaceutical Design* 1997; **3**: 59-84
8 **Vlahcevic ZR**, Pandak WM, Stravitz RT. Regulation of bile acid biosynthesis. *Gastroenterol Clin North Am* 1999; **28**: 1-25, v
9 **Li AP**. Roque MA, Beck DJ, Kaminski DL. Isolation and culturing of hepatocytes from human livers. *J Tiss Cult Meth* 1992; **14**: 139-146
10 **Björkhem I**, Falk O. Assay of the major bile acids in serum by isotope dilution-mass spectrometry. *Scand J Clin Lab Invest* 1983; **43**: 163-170
11 **Einarsson C**, Ellis E, Abrahamsson A, Ericzon BG, Bjorkhem I, Axelson M. Bile acid formation in primary human hepatocytes. *World J Gastroenterol* 2000; **6**: 522-525
12 **Ellis E**, Axelson M, Abrahamsson A, Eggertsen G, Thörne A, Nowak G, Ericzon BG, Björkhem I, Einarsson C. Feedback regulation of bile acid synthesis in primary human hepatocytes: evidence that CDCA is the strongest inhibitor. *Hepatology* 2003; **38**: 930-938
13 **Angelin B**, Einarsson K, Leijd B. Bile acid metabolism in hypothyroid subjects: response to substitution therapy. *Eur J Clin Invest* 1983; **13**: 99-106
14 **Sauter G**, Weiss M, Hoermann R. Cholesterol 7 alpha-hydroxylase activity in hypothyroidism and hyperthyroidism in humans. *Horm Metab Res* 1997; **29**: 176-179
15 **Galman C**. Modulation of bile acid and cholesterol metabolism in health and diease [Doctoral]. Stockholm: Karolinska Institute, 2004: 1-35
16 **Wang DP**, Stroup D, Marrapodi M, Crestani M, Galli G, Chiang JY. Transcriptional regulation of the human cholesterol 7 alpha-hydroxylase gene (CYP7A) in HepG2 cells. *J Lipid Res* 1996; **37**: 1831-1841
17 **Drover VA**, Wong NC, Agellon LB. A distinct thyroid hormone response element mediates repression of the human cholesterol 7alpha-hydroxylase (CYP7A1) gene promoter. *Mol Endocrinol* 2002; **16**: 14-23
18 **Crestani M**, Stroup D, Chiang JY. Hormonal regulation of the cholesterol 7 alpha-hydroxylase gene (CYP7). *J Lipid Res* 1995; **36**: 2419-2432
19 **Hylemon PB**, Gurley EC, Stravitz RT, Litz JS, Pandak WM, Chiang JY, Vlahcevic ZR. Hormonal regulation of cholesterol 7 alpha-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. *J Biol Chem* 1992; **267**: 16866-16871
20 **Andersson U**, Yang YZ, Björkhem I, Einarsson C, Eggertsen G, Gåfvels M. Thyroid hormone suppresses hepatic sterol 12alpha-hydroxylase (CYP8B1) activity and messenger ribonucleic acid in rat liver: failure to define known thyroid hormone response elements in the gene. *Biochim Biophys Acta* 1999; **1438**: 167-174
21 **Björkhem I**, Araya Z, Rudling M, Angelin B, Einarsson C, Wikvall K. Differences in the regulation of the classical and the alternative pathway for bile acid synthesis in human liver. No coordinate regulation of CYP7A1 and CYP27A1. *J Biol Chem* 2002; **277**: 26804-26807
22 **Stravitz RT**, Vlahcevic ZR, Russell TL, Heizer ML, Avadhani NG, Hylemon PB. Regulation of sterol 27-hydroxylase and an alternative pathway of bile acid biosynthesis in primary cultures of rat hepatocytes. *J Steroid Biochem Mol Biol* 1996; **57**: 337-347

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LIVER CANCER

Cross-species hybridization of woodchuck hepatitis virus-induced hepatocellular carcinoma using human oligonucleotide microarrays

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Abstract

AIM: To demonstrate the feasibility of using woodchuck samples on human microarrays, to provide insight into pathways involving positron emission tomography (PET) imaging tracers and to identify genes that could be potential molecular imaging targets for woodchuck hepatocellular carcinoma.

METHODS: Labeled cRNA from woodchuck tissue samples were hybridized to Affymetrix U133 plus 2.0 GeneChips®. Ten genes were selected for validation using quantitative RT-PCR and literature review was made.

RESULTS: Testis enhanced gene transcript (BAX Inhibitor 1), alpha-fetoprotein, isocitrate dehydrogenase 3 (NAD+) beta, acetyl-CoA synthetase 2, carnitine palmitoyltransferase 2, and N-myc2 were up-regulated and spermidine/spermine N1-acetyltransferase was down-regulated in the woodchuck HCC. We also found previously published results supporting 8 of the 10 most up-regulated genes and all 10 of the 10 most down-regulated genes.

CONCLUSION: Many of our microarray results were validated using RT-PCR or literature search. Hence, we believe that woodchuck HCC and non-cancerous liver samples can be used on human microarrays to yield meaningful results.

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Key words: Cross-species hybridization; Gene expression; Woodchuck hepatitis virus; Hepatocellular carcinoma; Woodchuck; *Marmota monax*

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INTRODUCTION

Patients with chronic hepatitis originating from infection with hepatitis B virus (HBV) are among those with the highest risk for hepatocellular carcinoma (HCC). The role of HBV in hepatocarcinogenesis has been studied by many investigators, but still not entirely understood. The first case of hepatocellular adenomas in a woodchuck was reported by Fox in 1912[1]. The model of hepatitis-induced HCC was later described by Summers in 1978[2] and is now an accepted animal model for studying HBV-induced HCC[3]. We have performed PET imaging with various positron-emitting radionuclide-labeled imaging tracers involved in the woodchuck hepatitis virus (WHV)-induced HCC animal model (details of the imaging study will be reported separately). Early studies in this project include investigating the genetic basis of pathways involving the tracers and looking for new genetic targets for molecular imaging.

In the past decade, microarray experiments have paved the way for thorough studies of gene expression. To date there has been no commercially available microarray to study gene expression in the woodchuck largely because the genome of this species has not been sequenced and it is not among the common animal models. Cross-species hybridization analysis is one of the methods to investigate genetic regulation of unusual species. Some authors have succeeded in using porcine samples on human Affymetrix GeneChips®[4,5]. One group has demonstrated the feasibility of using normal woodchuck samples on commercially available human nylon membrane arrays that have since been discontinued[6].

To the best of our knowledge, there have been no studies that used woodchuck samples on human

Table 1 RT-PCR primer and probe sequences for select genes

Target gene	Forward primer sequence	Reverse primer sequence	Reporter probe sequence
Testis enhanced gene transcript (BAX inhibitor 1) (TEGT)	GCCACCTGTAATGTGTGCAAAG	ACTTTCCCAGTTTGACTTCCTCTTC	CTCCACCCCTCATGTCC
Acetyl-CoA synthetase 2 (thiokinase) (ACAS2)	TGCTGAGGACCCACTCTTCAT	AGCATGTAACCGCCAACTGT	CCACAGGCAAACCCA
Alpha-fetoprotein (AFP)	AGGCTGTCATTGCAGATTTCTCT	GGACCCTCTTCTGCAAAGCA	CTGGCAGCATGTCTCC
C-myc (CMYC)	CAGAAGGTCAGAATCGGTGTCA	AGGACCAGTGGGCTGTGA	CCACAGCAAACCTC
Hexokinase (glucokinase regulatory protein) (GCKR)	ATGCTGCAGCGGTTCTCT	GGATCGCTTGGAGGAGACTCT	AAGGCCCGATGCATTG
Isocitrate dehydrogenase 3 (NAD+) beta (IDH3B)	TCTCAGCGGATTGCAAAGTTTG	CTTGTGGACAGCTGTGACCTT	CCCGCCCCTTCTTG
Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1)	GCGGGATGACACAGCAAAA	TCTTTCTGGAAATGTGAGTTATCCTTCTG	TCTGAAAATCTGAATATCCC
Spermidine/spermine N1-acetyltransferase (SSAT)	TTTCATGCAACACTTGGTCTCTCT	CACTGGACTCCGGAAGGTAAC	CCCTCACCCAATCCAG
Carnitine palmitoyltransferase II (CPT2)	TGCCTATTCCCAAACTTGAAGACA	TCTTCCTGAACTGGCCATCATTC	TCAATGCACAGAAACCT
N-myc 2 (NMYC2)	GGACGCGCTGAGTGGAT	GCTCTCGTTCGGCTCCAA	TTCCCAGGAGACCCC

oligonucleotide arrays to explore differential gene expression in non-cancerous woodchuck liver versus HCC. In this study, we analyzed the woodchuck gene expression using human Affymetrix GeneChips® with validation of differential expression of selected genes with quantitative RT-PCR and literature search.

MATERIALS AND METHODS

RNA isolation

All animals received humane care and the study protocol complies with the institutional guidelines. Woodchucks were euthanized after PET imaging and the livers were immediately removed from the animal. A pathologist separated non-cancerous liver and HCC. Tissues were snap-frozen in liquid nitrogen and stored at -80℃. RNA was extracted with RNeasy Midi Kit from Qiagen (Valencia, CA) according to the recommended protocol. An RNA integrity number (RIN) was found using a Bioanalyzer 2100 from Agilent (Palo Alto, CA).

Preparation of cRNA and microarray hybridization

RNA analyses were performed in the Gene Expression Array Core Facility at Case Western Reserve University. cRNA was prepared and hybridized to Affymetrix Human U133 plus 2.0 GeneChips® (Santa Clara, CA) according to the manufacturer's instructions. Microarray results were delivered in the form of cab files.

Microarray data analysis

Two sample *t* tests of quality control data were performed using R 2.0.1 statistical software. Hybridization results were analyzed using GeneChip® Operating Software (GCOS) 1.2 from Affymetrix (Santa Clara, CA). All microarrays were scaled to the same average signal intensity so that they could be compared to one another. Microsoft Excel and Access (Redmond, WA) were used to screen the data for significant results. Significantly changed genes were found using a modified version of the criteria outlined for a Comparison Analysis in the GeneChip® Expression Analysis: Data Analysis Fundamentals Manual from Affymetrix (Santa Clara, CA). The only criteria modified was to include signal fold changes for genes that were up-regulated by at least 1.5 or down-regulated by at least -1.5 in the HCC compared with the non-cancerous tissues.

RT-PCR

Available sequence information for woodchuck genes was found in the nucleotide database in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). These sequences were put into Applied Biosystems (Foster City, CA) for determination and production of optimal Custom Taqman® Gene Expression Assays. The Taqman® primer and probe sequences for our genes of interest are listed in Table 1. The Taqman® Assays and total RNA were given to the Gene Expression Array Core Facility. Assays were run in triplicate in a 15 μL reaction volume in a 384-well plate using a PRISM® 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA). The RT-PCR assay consisted of a 2-minute incubation at 50℃, a 10-minute incubation at 95℃ followed by 40 cycles of a 15-second incubation at 95℃, and then a 1-minute incubation at 60℃. Results were provided to us as values relative to a baseline sample. HCC was compared with the non-cancerous baseline sample from each animal, and > 2.5 or < 2.5 fold gene expression was considered up- or down-regulated, respectively.

RESULTS

Quality control

The first step in determining the usefulness of the results from a microarray study is to examine the quality control data. Table 2 shows some of RNA and microarray quality control data that we examined in this study. Two sample *t* tests were performed to see if there were differences between normal liver samples and HCC samples in any of the categories listed in Table 2. The "Percentage of

Table 2 Quality control data for cross-species hybridization

Samples	RNA integrity number (max = 10.0)	Average background	Noise	Percentage of genes called present	Scaling factor (scaled to 15)
W904Nb	7.5	33.750	1.00	9.0	2.959
W361N	7.8	34.170	0.97	9.5	2.970
W380N	9.7	32.089	0.93	9.9	2.955
W904T	9.8	36.880	1.07	10.3	2.206
W361T	9.2	35.720	1.05	10.5	2.355
W380T	9.9	31.610	0.94	10.8	2.463

Genes Called Present" and the "Scaling Factor" showed a statistically significant difference ($P < 0.05$) between the normal and the tumor samples. Housekeeping control and spiked control genes were qualitatively examined to have similar signal values and similar 3'-to-5' ratios across all of the samples.

Significantly changed genes

A number of the genes was found to be significantly changed in the HCC tissues compared with the normal portion of the liver from the same animal (Table 3). Sixty-six genes were found significantly changed in all three animals and 286 genes changed in 2 out of the 3 animals. Some genes with multiple probes on the microarray were also markedly changed. Of the 18 gene probes that were down-regulated in all 3 tumors, 5 presented metallothioneins, 3 for early growth response 1, and 2 for spermidine/spermine N1-acetyltransferase. Five genes were up-regulated in 2 probes in all 3 tumors: H2A histone family member Z, F-box protein 9, a disintegrin and metalloproteinase domain 10, butyrate-induced transcript 1, and ribophorin II.

We studied further into the 10 most up- and down-regulated genes that were common to all three HCC versus normal liver sample pairs (Table 4). Eight of the top 10 up-regulated genes found previously in literature are in agreement with our findings[7-14]. We were unable to find any publication describing the up-regulation in any cancer for 2 of these genes, one of which was an open reading frame, but found published data supporting all of the 10 most down-regulated genes[15-21].

One group used normal human and normal woodchuck liver samples on human nylon membrane arrays[6]. To compare our results to theirs, we looked into 48 genes which were found to be commonly expressed in both normal human and normal woodchuck livers. We used 2 normal woodchuck liver samples from uninfected woodchucks on the Affymetrix human U133 plus 2.0 GeneChip® and found 21 of the 48 previously reported genes to be expressed. We were unable to find any probes on the GeneChip® for 3 of the 48 genes. We also explored the normal portion of the liver from 3 woodchucks with HCC and found 22 of the 48 genes were present in all 3 animals, 26 were present in 2 animals and 34 were present in at least 1 animal. Eighteen genes were present in both the normal liver from an uninfected woodchuck and the non-cancerous liver samples from all 3 woodchucks with HCC.

Table 3 Significantly changed genes

Sample pair (tumor *vs* normal)	Number of up-regulated genes[1]	Number of down-regulated genes[1]	Total number of changed genes
W904	596	205	801
W361	359	242	601
W380	215	81	296
W904, W361	142	47	189
W904, W380	66	3	69
W361, W380	19	9	28
W904, W361, W380	48	18	66

[1]The data is presented as HCC *vs* normal portion of the liver sample where the normal sample is the baseline.

RT-PCR

Ten genes were selected for RT-PCR analysis. Two genes were selected to validate the microarray analysis because the microarray analyses demonstrated they were significantly changed (TEGT and SSAT). RT-PCR was also performed on other genes due to their role in metabolic pathways and hepatocellular carcinogenesis. The RT-PCR results along with the corresponding microarray results are shown in Table 5. It should be noted that the terms "no change" and "undetermined" are not the same. A sample with no change shows similar expression in the normal and tumor samples, whereas the undetermined means that expression could not be measured in either the normal or tumor sample using RT-PCR.

DISCUSSION

PET imaging allows clinicians to view *in vivo* distribution and uptake of a radiolabeled compound, or imaging tracer. It is a useful diagnostic tool when a tracer is used that is highly specific to its target. We investigated the performance of various tracers and their metabolism regulated at multiple levels in the woodchuck model of hepatitis virus-induced HCC. We conducted a microarray study to explore the transcriptional regulation of interesting genes.

There is a logical rationale to explain the disparity in quality control data between normal and tumor samples. In the normal tissue, there were fewer genes called "Present" and a larger scaling factor as shown in Table 2. More genes were up-regulated in the tumors than were down-regulated. Therefore, more genes were expressed in the tumors compared with the non-cancerous portion of the liver, hence the larger 'Percentage of Genes Called Present'. The fact that fewer genes were called 'Present' in the non-cancerous samples is the likely reason for the lower overall average signal intensity, which would also mean that a larger scaling factor is needed to reach a target value compared with the tumor samples.

To determine if our microarray results were consistent with other studies, we compared our results using normal woodchuck liver samples from 2 uninfected animals on Affymetrix U133 plus 2.0 GeneChips® to the results of another group that used similar uninfected woodchuck

Table 4 Top 10 up- and down-regulated genes in common on all three tumor/normal sample pairs

Fold change[1]	Gene title	Gene symbol	Location	Process	Agreement
14.3294	Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	10q23-q24	Fatty Acid Biosynthesis	[7]
11.6297	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	ELOVL6	4q25	Fatty Acid Biosynthesis	[8]
7.7381	Chromosome 9 open reading frame 41	C9orf41	9q21.13		none
5.1036	Cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51A1	7q21.2-q21.3	Electron Transport/ Cholesterol Biosynthesis	[9]
4.1746	H2A histone family, member Z	H2AFZ	4q24	DNA Replication	[10]
3.4227	H2A histone family, member Z	H2AFZ	4q24	DNA Replication	[10]
3.3977	TATA box-binding protein-like protein 1, TBP-like 1	TBPL1	6q22.1-q22.3	Transcription	none
2.9307	Diacylglycerol O-acyltransferase homolog 2 (mouse)	DGAT2	11q13.5	Triacylglycerol biosynthesis	[11]
2.8134	ARP2 actin-related protein 2 homolog (yeast)	ACTR2	2p14	Protein Binding/Structural Molecule Activity	[12,13]
2.8134	Calcium binding protein P22	CHP	15q13.3	Potassium Ion Transport/Small GTPase Mediated Signal Transduction	[14]
-3.6151	Metallothionein 1H	MT1H	16q13	Metal Ion Binding	[15,16]
-3.6353	Metallothionein 2A	MT2A	16q13	Metal Ion Binding	[15,16]
-3.9071	Metallothionein 1X	MT1X	16q13	Metal Ion Binding	[15,17]
-4.1870	EST similar to early growth response 1				[18,19]
-4.5774	Metallothionein 1F (functional)	MT1F	16q13	Metal Ion Binding	[15,16]
-4.9625	Early growth response 1	EGR1	5q31.1	Regulation of Transcription	[18,19]
-5.5956	Metallothionein 1F (functional)	MT1F	16q13	Metal Ion Binding	[15,16]
-5.7266	Sprouty homolog 2 (Drosophila)	SPRY2	13q31.1	Cell-Cell Signaling/ Development/ Organogenesis/ Regulation of Signal Transduction	[20]
-10.3842	Early growth response 1	EGR1	5q31.1	Regulation of Transcription	[18,19]
-24.2139	Insulin-like growth factor binding protein 2, 36 kDa	IGFBP2	2q33-q34	Regulation of Cell Growth	[21]

[1]This is the average fold change for all 3 HCC *vs* normal sample pairs.

Table 5 RT-PCR and microarray results

		W904		W361		W380	
Gene	Process	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray
Testis enhanced gene transcript (BAX inhibitor 1) (TEGT)	Negative regulation of apoptosis	No change	Up	Up	Up	Up	Up
Spermidine/spermine N1-acetyltransferase (SSAT)	Polyamine metabolism	Down	Down	Down	Down	No change	Down
Acetyl-CoA synthetase 2 (thiokinase) (ACAS2)[1]	Metabolism, lipid biosynthesis	Up	Up	Up	Up	Up	No change
Alpha-fetoprotein (AFP)	Immune response, transport	Up	No change	Up	No change	Up	Up
Isocitrate dehydrogenase 3 (NAD+) beta (IDH3B)	Tricarboxylic acid cycle	Up	Up	Undetermined	No change	Up	Up
Carnitine palmitoyltransferase II (CPT2)	Lipid metabolism, fatty acid metabolism, transport	Up	No change	Up	No change	No change	No change
N-myc 2 (NMYC2)	Unknown	Undetermined	N/A	Up	N/A	Undetermined	N/A
C-myc (CMYC)[2]	Regulation of transcription, DNA-dependent	Up	Up	Down	Up	No change	No change
Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1)	Lipid metabolism, fatty acid metabolism	Down	Down	Undetermined	Down	Up	No change
Hexokinase (GCKR)[3]	Carbohydrate metabolism	Undetermined	No change	Undetermined	Up	Up	No change

[1]RT-PCR results are for ACAS2, microarray results are for a hypothetical protein similar to ACAS2. [2]RT-PCR results are for c-myc, microarray results are for a c-myc binding protein and a downstream target. [3]RT-PCR results are for glucokinase regulatory protein, microarray results are for an EST similar to hexokinase domain containing 1.

liver samples on human nylon membrane arrays[6]. Using the standard microarray protocol according to the manufacturer's instructions, we have demonstrated a large overlap of results from the two studies as well as a high degree of similarity between the normal liver tissue from uninfected woodchucks and those of woodchucks with

HCC.

We found supporting data for 8 of the top 10 up-regulated genes and all 10 of the top 10 down-regulated genes (Table 4). The top two up-regulated genes (steroyl-CoA desaturase and elongation of long chain fatty acids, family member 6) are involved in fatty acid biosynthesis, which suggests that fatty acid synthesis is up-regulated in HCC compared with the surrounding non-cancerous liver tissues in woodchucks.

Due to the limited number of woodchuck genes that have been sequenced (fewer than 600), we could not perform RT-PCR on all of the genes of interest, and in some cases the sequence was not available for the exact gene. FACVL1, AFP, IDH3B, CPT2, and SSAT had specific probes on the microarray; ACAS2, CMYC, GCKR, and TEGT were similar to what we had microarray probes for; and there was no probe on the microarray for NMYC2. The microarray results showed expression of a hypothetical protein similar to the 5' region of ACAS2, a c-myc binding protein, a c-myc downstream target, an EST similar to hexokinase domain containing 1, and an EST similar to TEGT. Of the 66 probes on the microarrays that significantly changed in all 3 pairs of samples, we validated 2 genes with available sequence information. TEGT was up-regulated and SSAT was down-regulated in the tumors as shown by both the microarray and RT-PCR analysis. TEGT is a negative regulator of apoptosis and has been shown to be up-regulated in cancer cells[22] and SSAT, which is the rate limiting step in polyamine catabolism, has been shown to be induced at the transcriptional level to sensitize tumors for adjuvant treatment[23].

We also investigated genes involved in carcinogenesis and various metabolic pathways. IDH3B, the rate-limiting enzyme in the tricarbocylic acid cycle, was up-regulated in 2 probes in the same 2 tumors and expression validated with RT-PCR. A hypothetical protein similar to ACAS2, which catalyzes the conversion of acetate to acetyl-CoA for use in many metabolic and biosynthetic pathways, was up-regulated in 2 tumors according to the microarray results and shown to be up-regulated in all 3 tumors with RT-PCR. ACAS2 is of particular relevance because of the usage of the PET imaging tracer [^{11}C]-Acetate. Currently, it is not entirely known what pathways are responsible for the high uptake of acetate which can be seen in HCC compared with surrounding normal liver tissues in the PET images. We have demonstrated high up-regulation of the enzyme that catalyzes the formation of acetyl-CoA from acetate, so we hypothesize that this may be the first step involving [^{11}C]-Acetate.

An EST similar to hexokinase was up-regulated in 1 tumor according to the microarray results, and glucokinase regulatory protein (GCKR) was up-regulated in a different tumor based on RT-PCR. Sequence information for the woodchuck glucokinase gene was not available, so RT-PCR was performed on a GCKR, a negative regulator of glucokinase. Glucokinase is important for its role in PET imaging of [^{18}F]-fluorodeoxyglucose (FDG). Glucokinase catalyzes the phosphorylation of FDG, thereby trapping it in the cell according to previous kinetic modeling data[24]. Up-regulation of glucokinase or down-regulation of GCKR would be expected to result in higher uptake of FDG in HCC compared with the non-cancerous liver tissues. However, our results were not conclusive and future work will require sequencing of the woodchuck hexokinase gene or examination of glucokinase regulation at the protein level.

CPT2, a protein that facilitates transport of long-chain fatty acids to the inner mitochondrial membrane for fatty acid metabolism, did not show any change according to the microarray results, but was found to be up-regulated in 2 of the 3 HCCs using RT-PCR. In the target sequence used to create the probes on the human microarray, there is a 436 residue overlap with 40.4% homology between the microarray CPT2 target sequence and the woodchuck CPT2 sequence used for RT-PCR. The lack of homology between the sequences might be the reason for not obtaining significant microarray results.

AFP, a serum marker for HCC in humans[25], was up-regulated in one tumor and showed no change in the other two on the microarrays, while it was up-regulated in all three tumors as measured by using RT-PCR. NMYC2, which was found highly expressed in 60% of woodchuck HCCs[26], was up-regulated in one tumor by using RT-PCR.

These results from using human Affymetrix GeneChips® provide a part of the foundation for our imaging work to explore the woodchuck model of virus-induced HCC. Future work will include more investigations of the regulation of pathways involving PET imaging tracers using enzyme assays and immunohistochemistry. Additional efforts will be focused on developing new radiolabeled antibodies and/or ligands for potential imaging targets found to be up-regulated in woodchuck HCC compared with surrounding non-neoplastic woodchuck hepatic tissues.

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REFERENCES

1 **Fox H**. Observations upon neoplasms in wild animals in the Philadelphia Zoological Garden. *J Pathol Bacteriol* 1912; **17**: 217-231

2 **Summers J**, Smolec JM, Snyder R. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc Natl Acad Sci USA* 1978; **75**: 4533-4537

3 **Tennant BC**, Toshkov IA, Peek SF, Jacob JR, Menne S, Hornbuckle WE, Schinazi RD, Korba BE, Cote PJ, Gerin JL. Hepatocellular carcinoma in the woodchuck model of hepatitis B virus infection. *Gastroenterology* 2004; **127**: S283-S293

4 **Shah G**, Azizian M, Bruch D, Mehta R, Kittur D. Cross-species comparison of gene expression between human and porcine tissue, using single microarray platform--preliminary results. *Clin Transplant* 2004; **18** Suppl 12: 76-80

5 **Ji W**, Zhou W, Gregg K, Yu N, Davis S, Davis S. A method for cross-species gene expression analysis with high-density oligonucleotide arrays. *Nucleic Acids Res* 2004; **32**: e93

6 **Rinaudo JA**, Gerin JL. Cross-species hybridization: characterization of gene expression in woodchuck liver using human membrane arrays. *J Med Virol* 2004; **74**: 300-313

7 **Li J**, Ding SF, Habib NA, Fermor BF, Wood CB, Gilmour RS. Partial characterization of a cDNA for human stearoyl-CoA desaturase and changes in its mRNA expression in some normal and malignant tissues. *Int J Cancer* 1994; **57**: 348-352

8 **Sawada H**, Takami K, Asahi S. A toxicogenomic approach to drug-induced phospholipidosis: analysis of its induction mechanism and establishment of a novel in vitro screening system. *Toxicol Sci* 2005; **83**: 282-292

9 **Tönnies H**, Lage H. Chromosomal imbalances associated with drug resistance and thermoresistance in human pancreatic carcinoma cells. *Eur J Cell Biol* 2004; **83**: 591-601

10 **Hatch CL**, Bonner WM. The human histone H2A.Z gene. Sequence and regulation. *J Biol Chem* 1990; **265**: 15211-15218

11 **Kurowska EM**, Manthey JA, Casaschi A, Theriault AG. Modulation of HepG2 cell net apolipoprotein B secretion by the citrus polymethoxyflavone, tangeretin. *Lipids* 2004; **39**: 143-151

12 **Otsubo T**, Iwaya K, Mukai Y, Mizokami Y, Serizawa H, Matsuoka T, Mukai K. Involvement of Arp2/3 complex in the process of colorectal carcinogenesis. *Mod Pathol* 2004; **17**: 461-467

13 **Abraham MT**, Kuriakose MA, Sacks PG, Yee H, Chiriboga L, Bearer EL, Delacure MD. Motility-related proteins as markers for head and neck squamous cell cancer. *Laryngoscope* 2001; **111**: 1285-1289

14 **Pang T**, Wakabayashi S, Shigekawa M. Expression of calcineurin B homologous protein 2 protects serum deprivation-induced cell death by serum-independent activation of Na+/H+ exchanger. *J Biol Chem* 2002; **277**: 43771-43777

15 **Cherian MG**, Jayasurya A, Bay BH. Metallothioneins in human tumors and potential roles in carcinogenesis. *Mutat Res* 2003; **533**: 201-209

16 **Okabe H**, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001; **61**: 2129-2137

17 **Garrett SH**, Sens MA, Shukla D, Flores L, Somji S, Todd JH, Sens DA. Metallothionein isoform 1 and 2 gene expression in the human prostate: downregulation of MT-1X in advanced prostate cancer. *Prostate* 2000; **43**: 125-135

18 **Huang RP**, Fan Y, de Belle I, Niemeyer C, Gottardis MM, Mercola D, Adamson ED. Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. *Int J Cancer* 1997; **72**: 102-109

19 **Calogero A**, Arcella A, De Gregorio G, Porcellini A, Mercola D, Liu C, Lombari V, Zani M, Giannini G, Gagliardi FM, Caruso R, Gulino A, Frati L, Ragona G. The early growth response gene EGR-1 behaves as a suppressor gene that is down-regulated independent of ARF/Mdm2 but not p53 alterations in fresh human gliomas. *Clin Cancer Res* 2001; **7**: 2788-2796

20 **McKie AB**, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R, Gnanapragasam VJ, Robson CN, Leung HY. Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. *Oncogene* 2005; **24**: 2166-2174

21 **Gong Y**, Cui L, Minuk GY. The expression of insulin-like growth factor binding proteins in human hepatocellular carcinoma. *Mol Cell Biochem* 2000; **207**: 101 - 104

22 **Grzmil M**, Thelen P, Hemmerlein B, Schweyer S, Voigt S, Mury D, Burfeind P. Bax inhibitor-1 is overexpressed in prostate cancer and its specific down-regulation by RNA interference leads to cell death in human prostate carcinoma cells. *Am J Pathol* 2003; **163**: 543-552

23 **Wang Y**, Devereux W, Stewart TM, Casero RA Jr. Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N(1)-acetyltransferase gene. *J Biol Chem* 1999; **274**: 22095-22101

24 **Phelps ME**, Huang SC, Hoffman EJ, Selin C, Sokoloff L, Kuhl DE. Tomographic measurement of local cerebral glucose metabolic rate in humans with (F-18)2-fluoro-2-deoxy-D-glucose: validation of method. *Ann Neurol* 1979; **6**: 371-388

25 **Johnson PJ**. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis* 2001; **5**: 145-159

26 **Fourel G**, Trepo C, Bougueleret L, Henglein B, Ponzetto A, Tiollais P, Buendia MA. Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumours. *Nature* 1990; **347**: 294-298

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LIVER CANCER

Hepatectomy for huge hepatocellular carcinoma in 634 cases

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Abstract

AIM: To clarify the safety and feasibility of hepatectomy for huge hepatocellular carcinoma (HCC).

METHODS: A total of 4765 patients with HCC operated at Tongji Hospital were retrospectively studied, of them, 780 patients had huge HCC (10 cm or more in diameter). Hepatectomy was carried out on 634 patients (81.2%). The majority of the liver resection were major resections, and combined resection of the adjacent organs or structures was common (17.2%). The liver resection was combined with portal vein thrombectomy in 139 patients (21.9%).

RESULTS: Postoperative complications were common (26.8%) and required another laparotomy to prevent the complications in 5 patients (0.8%). The 30-d mortality was 2.2%. The main causes of postoperative deaths were liver failure (n = 9), postoperative bleeding (n = 4) and septic complication (n = 1). The 3-, 5- and 10-year survival rates after liver resection were 35.1%, 18.2% and 3.5%, respectively.

CONCLUSION: Hepatectomy for huge HCC is safe and effective. It should be used to treat patients with low surgical risks and resectable tumours.

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Key words: Hepatectomy; Huge hepatocellular

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an annual occurrence of at least one million new cases[1-3].

Hepatic resection is the mainstay of treatment for resectable tumours because of the proven impact of adequate tumor removal on prognosis. Recent improvements in perioperative management have made partial hepatectomy safe.

Growing indications are related to the resection extent and the approach of surgery in patients with HCC, and the maximum prognosis benefit can be combined with optimal safety. Therefore, surgeons should balance the benefits of oncological clearance against the risks of inadequate parenchymal preservation after liver resection.

A non-cirrhotic liver can tolerate up to 80% liver resection. The regenerative capacity of the liver enables a functional compensation within a few weeks and regeneration to roughly 75% of the preoperative liver volume within 1 year. Such a favourable outcome, however, cannot be extrapolated to cirrhotic liver resection which is commonly carried out for HCC.

Hepatectomy for huge HCC is associated with a higher operative morbidity and mortality because of the extent of liver resection, and a histological margin of over 1 cm is usually recommended to cut down on intrahepatic tumor recurrence[4-6]. Of the many prognostic factors associated with long-term survival, the size of tumor has a positive association after 'curative' resection for HCC[6-10]. The aim of this retrospective study was to find out whether liver resection for huge HCC (tumor bigger than 10 cm in diameter) is efficacious and safe.

MATERIALS AND METHODS

A retrospective study was done on 4765 patients with HCC who were treated surgically at the Tongji Hospital, Wuhan, China from January 1972 to December 2002, and 780 (16.8%) patients had huge HCC over 10 cm in diameter. Liver resection was carried out on 634 patietns (81.2%).

The criteria for liver resection for huge HCC with curative intention were good general condition of the patient, good cardiopulmonary and renal functions, Pugh-Child's A and B, normal indocyanine green test, tumor confined to one lobe of the liver, and no extrahepatic spread of disease with the exception of local invasion to

Table 1 Demographic data of 634 patients with huge hepatocelular carcinoma who underwent liver resection

Sex (male:female)	563:71
Age (yr)[1]	39 ± 9.5
Total bilirubin (μmol/L)[1]	12.5 ± 2.8
Alanine aminotransferase (U/L)	36 ± 8.5
Albumin (mmol/L)[1]	3.8 ± 0.15
Prothrombin times (seconds prolonged)[1]	1.48 ± 0.8
Maximum size of tumor (cm)[1]	14.1 ± 2.6
Pugh-Child's grading, *n* (%)	
A	559 (88.2%)
B	75 (11.8%)
C	0
Alpha fetoprotein ⩾ 400 ng/mL	310 (58.9%)[2]
HBsAg positive	469 (74.0%)
Satellite nodules	139 (26.5%)
Tumor thrombi in portal vein	139 (21.9%)
Associated cirrhosis	547 (86.3%)

[1] mean ± SD. [2] number of patient studied = 498.

Table 2 Type of operation (*n* = 634)

Type of liver resection	*n*	%
Resection of right three lobes	30	4.8
Resection of right half	44	6.9
Resection of left three lobes	39	6.2
Resection of left half	63	9.9
Segmentectomy 4, 5, 6	33	5.2
Segmentectomy 4, 5, 8	27	4.2
Segmentectomy 5, 6, 7	87	13.7
Segmentectomy 2, 3	128	20.2
Non-anatomical resections	183	28.9
Total	634	100
Combined organ resection		
Partial diaphragamectomy	12	1.9
Right adrenal gland resection	9	1.4
Distal gastrectomy	5	0.8
Transverse colectomy	11	1.7
Splenectomy	31	4.9
Splenectomy + devascularization	41	6.5
Portal vein thrombectomy	139	21.9
Total	248	39.1

adjacent organs.

Liver resection for huge liver tumours was performed as previously described[11,12]. In brief, we routinely used the Pringle's manoeuvre to occlude the hepatic inflow. We divided the porta hepatis pedicle supplying the side of the liver to be resected as described by Launois[13]. We then transected the liver parenchyma using finger-fracture technique with a haemostat along the plane of transection. The hepatic vein draining the part of the liver to be resected was ligated and divided intrahepatically. Then the portion of the liver to be resected by division of the ligaments was mobilized. This method of liver resection prevented laceration of the major vessels during mobilization of the liver, avoided rupturing of the huge tumor due to excessive traction, and excessive bleeding from the venous collaterals due to cirrhosis and portal hypertension by leaving mobilization of the liver near the end of liver resection. Oncological principles of ligating the vessels prior to manipulation of tumor were followed to diminish shedding of tumor cells into the blood stream.

RESULTS

Of the 780 patients with huge HCC of over 10 cm in diameter treated surgically, 634 (81.2%) underwent liver resection. The remaining 146 patients had unresectable tumors at laparotomy. The reasons for unresectability were: tumours too extensively involving the liver (*n* = 27), multiple secondary tumors (*n* = 56), severe liver cirrhosis (*n* = 44), and tumor infiltration into major vascular structures including the main portal vein, common hepatic artery or the hepatic veins (*n* = 19). These patients with unresectable HCC were treated with intraoperative transhepatic or transportal vein chemotherapy, or with chemotherapy *via* a totally implantable drug infusion system (*n* = 57), intraoperative microwave coagulation (*n* = 36), intraoperative cryotherapy (*n* = 45), or a biopsy of the liver tumor (*n* = 8).

The demographic data of the 634 patients who undergoing liver resection are shown in Table 1. Majority of the patients underwent major liver resection. The terminology of the liver resection used was included according to the recommendation by the International Hepato-Pancreato-Biliary Association. Combined resection of adjacent organs involving the tumor was common. A significant proportion of patient had tumor thrombi in the portal vein (Table 2).

The operative time was 98 ± 41 min, inflow occlusion time was 10.5 ± 2.7 min. Blood loss was 440 ± 250 mL and transfusion requirement was 480 ± 350 mL. Postoperative complications were common, such as reactive right pleural effusion (*n* = 135 , 21.4%), significant ascites (*n* = 30, 4.7%), bleeding from stress ulceration (*n* = 11, 1.7%), postoperative hemorrhage on the surface of liver (*n* = 7, 1.3%), infected wound (*n* = 10, 1.6%), bile leakage (*n* = 7, 1.1%) and intraabdominal abscess (*n* = 6, 0.9%). Relaparotomy to deal with the complications was required in 5 patients (0.8%). The 30-d mortality rate was 2.2%, 9 of 14 patients died of liver failure, 4 of 14 died of postoperative bleeding, and 1 died of septic complication. The 3-, 5- and 10-year survival rates were 35.1%, 18.2% and 3.5%, respectively (Figure 1).

Univariate analysis showed the following prognostic factors associated with significant improvement in survival: female sex, low serum alpha fetoprotein of less than 400 ng/mL, presence of tumor capsule, absence of extensive capsular infiltration by tumor, absence of tumor thrombus in main portal vein and satellite nodules (Table 3). On multivariate analysis, the significant prognostic factors for long-term survival were extensive capsular infiltration, tumor thrombus in portal vein, satellite nodules and intraoperative blood loss (Table 4).

DISCUSSION

HCC usually presents at a very late stage in its natural history because of the lack of symptoms in the early stage. The large size and the position of the liver behind the costal cartilages preclude the tumor from being readily palpable during early stage. Furthermore, the large functional reserve of the liver delays clinical presentation

Table 3 Univariate analysis on prognostic factors associated with long-term survival in patients with huge hepatocellular carcinoma undergoing liver resection

Variable	Patients (*n*)	Survival rates (%) 1 yr	2 yr	3 yr	5 yr	
Patients						
Sex Male	332	65.1	43.7	33.7	15.1	$P < 0.05$
Female	24	83.3	70.8	45.8	37.5	
Age < 50 yr	206	64.5	42.7	32.0	15.1	NS
⩾ 50 yr	150	68.7	46.3	38.0	18.7	
[1]AFP < 400 ng/mL	130	68.4	49.2	37.7	19.2	$P < 0.05$
⩾ 400 ng/mL	154	62.3	32.5	26.0	9.1	
Tumor size						
10-15 cm	281	71.5	46.6	39.5	17.4	$P < 0.05$
> 15 cm	75	46.7	41.3	16.0	0	
Capsule						
Positive	270	71.0	48.1	44.0	21.9	$P < 0.05$
Negative	86	48.8	37.2	4.7	0	
Extensive capsular infiltration						
Positive	224	67.4	40.6	39.3	12.1	$P < 0.05$
Negative	46	93.5	84.8	76.1	69.6	
Tumor thrombus in portal vein						
Positive	62	38.7	22.6	6.5	0	$P < 0.05$
Negative	294	72.1	50.3	40.5	20.1	
Satellite nodules						
Positive	87	36.8	23.0	12.6	0	$P < 0.01$
Negative	269	75.8	52.8	41.6	21.9	
Surgical resection margin						
⩽ 10 mm	251	64.1	40.2	34.3	13.9	NS
> 10 mm	105	71.4	58.1	35.2	22.9	
Intraoperative blood loss						
⩽ 800 mL	260	69.6	43.8	33.5	15.4	NS
> 800 mL	96	57.3	50.0	37.5	19.8	

[1]$n = 498$.

Table 4 Multivariate analysis on prognostic factors associated with long-term survival in patients with huge hepatocellular carcinoma undergoing liver resection ($n = 356$)

Factor (variable)	Coefficient of regression	Relative risk	Standard error	Wales statistic	*P*
Capsule					
Positive	-1.5152	1.3521	0.5078	9.543	< 0.05
Negative					
Extensive capsular infiltration					
Positive	-2.5237	1.4152	0.4035	10.321	< 0.05
Negative					
Tumor thrombus in portal vein					
Positive	-3.1012	2.4240	0.7122	7.543	< 0.05
Negative					
Satellite nodules					
Positive	-2.5158	1.7852	0.8055	7.257	< 0.05
Negative					
Intraoperative blood loss					
⩽ 800 mL	-5.5257	1.5023	0.7211	15.15	< 0.05
> 800 mL					

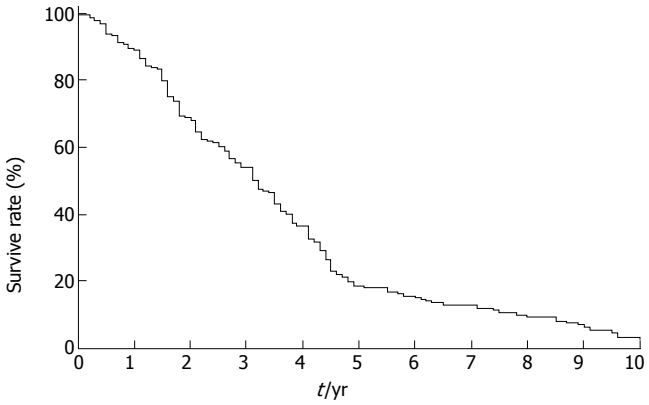

Figure 1 Survival curve for patients who underwent liver resection for huge hepatocellular carcinoma ($n = 634$). The 3-, 5-, and 10-yr survival rates were 35.1%,18.2% and 3.5%, respectively.

with functional disturbances of the liver. Some patients present with large tumors in the liver in their first visits.

The tumor size is a significant risk factor for intrahepatic and extrahepatic spread[14-16]. Whether the tumor's greatest diameter impacts on the disease-free interval and the overall survival remains unclear. Even when small HCCs are examined as a specific group, a better survival can still be observed with decreasing size of the tumor after resection[7,9]. The frequency of intrahepatic metastasis increases by almost a third between HCC smaller and larger than 5 cm and the rate of portal vein tumor thrombosis is almost doubled[15,16]. There is little information showing that patients with huge HCC over 10 cm in diameter without any extrahepatic metastases have long-term results after liver resection. Our study on 634 patients showed a 5-year survival of 18.2%. It is low when compared with reported series of patients with HCC unselected for the tumor size, the overall 5-year survival of these series ranges from 11% to 76% with a median around 30%, which might be due to the more advanced stage of cancer at the time of surgery.

One of the major concerns in operating on huge HCC is the operative mortality because of the extent of the liver resection, which is commonly involved in these huge tumors. The 30-d mortality of our patients was 2.2% which is low compared with that reported in the medical literature on HCC ranging from 0.5% to 21.5%, with a median of around 5% to 10%. Our low operative mortality is probably related to the surgeons' experience and careful case selection.

Intraopertive blood loss can adversely affect the perioperative mortality of patients undergoing liver resection[18-20]. There is evidence that blood transfusion may be associated with an increased risk of recurrence in patients operated for a hepato-biliary malignancy, due to impairment of the patient's immune response[21]. Our method of liver resection[11,12] can offer much to patients with huge HCC. As discussed previously, our technique not only cuts down the intraoperative bleeding, but also follows oncological principles. The other factor related to our good results is careful selection of patients. In cirrhotic liver resection, the onset of postoperative liver failure and mortality is related to the degree of preoperative liver failure[22] and the extent of non-tumor liver parenchyma removed[23]. An ideal candidate for liver resection in a patient with huge HCC is a patient with good liver function without ascites (Pugh-Child A).

Using univariate and multivariate analyses, we identified several prognostic factors, associated with a better long-term survival for patients with huge HCC after live resection. However, all these factors reflect the biologic

behavior of the tumor, and cannot be used to exclude patients from liver resection with an intention for a cure. They can be used as a guide to make decisions in balancing the risks of the operation against the potential benefits in a patient who has increased risks of mortality because of his general condition or borderline liver functions. In cirrhotic liver resection, resecting too generous a margin can result in a higher chance of postoperative liver failure. However, a negative histological margin is the only factor that the surgeon can control the long-term survival results. Therefore the tumor should be resected aiming at a 1cm gross margin although our study showed resection margin of above or below 1 cm had no impact on long-term survival provided that the histological margin was clear.

In conclusion, in carefully selected patients, liver resection in patients with HCC of 10 cm or more in diameter is safe.

REFERENCES

1 **Lau WY**. Management of hepatocellular carcinoma. *J R Coll Surg Edinb* 2002; **47**: 389-399

2 **Blumgart LH**, Leach KG, Karran SJ. Observations on liver regeneration after right hepatic lobectomy. *Gut* 1971; **12**: 922-928

3 **Miyagawa S**, Kawasaki S, Noike T, Nomura K, Kobayashi A, Shimada R, Imamura H. Liver regeneration after extended right hemihepatectomy in patients with hilar or diffuse bile duct carcinoma. *Hepatogastroenterology* 1999; **46**: 364-368

4 **Yamanaka N**, Okamoto E, Toyosaka A, Mitunobu M, Fujihara S, Kato T, Fujimoto J, Oriyama T, Furukawa K, Kawamura E. Prognostic factors after hepatectomy for hepatocellular carcinomas. A univariate and multivariate analysis. *Cancer* 1990; **65**: 1104-1110

5 **Sugioka A**, Tsuzuki T, Kanai T. Postresection prognosis of patients with hepatocellular carcinoma. *Surgery* 1993; **113**: 612-618

6 **Lehnert T**, Otto G, Herfarth C. Therapeutic modalities and prognostic factors for primary and secondary liver tumors. *World J Surg* 1995; **19**: 252-263

7 **Shirabe K**, Kanematsu T, Matsumata T, Adachi E, Akazawa K, Sugimachi K. Factors linked to early recurrence of small hepatocellular carcinoma after hepatectomy: univariate and multivariate analyses. *Hepatology* 1991; **14**: 802-805

8 The Liver Study Group of Japan. Primary liver cancer in Japan. *Cancer* 1990; **54**: 1747-1755

9 **Tang ZY**, Yu YQ, Zhou XD, Ma ZC, Yang R, Lu JZ, Lin ZY, Yang BH. Surgery of small hepatocellular carcinoma. Analysis of 144 cases. *Cancer* 1989; **64**: 536-541

10 **Kawano N**, Ohmori Y, Inoue S, Nagao T, Morioka Y. Long-term prognosis of surgical patients with hepatocellular carcinoma. *Cancer Chemother Pharmacol* 1989; **23** Suppl: S129-S132

11 **Chen XP**, Wu ZD, Qiu FZ. Right hemihepatectomy of directly tie off afferent and efferent hepatic vessels of the bearing-bearing liver. *Zhonghua Shiyong Waike Zazhi* 1999; **19**: 678-679

12 **Chen XP**, Wu ZD, Qiu FZ. Left hemiheptectomy of directly tie off afferent and efferent hepatic vessels of the bearing-bearing liver. *Dig Surg* 1999; 2: 69-70

13 **Launois B**. The intrahepatic Glissonian approach to liver resection. In : Blumgart LH, Fong Y (eds) Surgery of the Liver and Biliary Tract. London: WB Saunders, 2000: 1698-1703

14 The general rules for the clinical and pathological study of primary liver cancer. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129

15 **Yuki K**, Hirohashi S, Sakamoto M, Kanai T, Shimosato Y. Growth and spread of hepatocellular carcinoma. A review of 240 consecutive autopsy cases. *Cancer* 1990; **66**: 2174-2179

16 **Adachi E**, Maeda T, Kajiyama K, Kinukawa N, Matsumata T, Sugimachi K, Tsuneyoshi M. Factors correlated with portal venous invasion by hepatocellular carcinoma: univariate and multivariate analyses of 232 resected cases without preoperative treatments. *Cancer* 1996; **77**: 2022-2031

17 **Lau WY**. Primary hepatocellular carcinoma. In: Blumgart LH, Fong Y (eds) Surgery of the Liver and Biliary Tract. London: WB Saunders, 2000: 1423-1450

18 **Nagao T**, Inoue S, Goto S, Mizuta T, Omori Y, Kawano N, Morioka Y. Hepatic resection for hepatocellular carcinoma. Clinical features and long-term prognosis. *Ann Surg* 1987; **205**: 33-40

19 **Makuuchi M**, Takayama T, Gunvén P, Kosuge T, Yamazaki S, Hasegawa H. Restrictive versus liberal blood transfusion policy for hepatectomies in cirrhotic patients. *World J Surg* 1989; **13**: 644-648

20 **Shimada M**, Matsumata T, Akazawa K, Kamakura T, Itasaka H, Sugimachi K, Nose Y. Estimation of risk of major complications after hepatic resection. *Am J Surg* 1994; **167**: 399-403

21 **Rosen CB**, Nagorney DM, Taswell HF, Helgeson SL, Ilstrup DM, van Heerden JA, Adson MA. Perioperative blood transfusion and determinants of survival after liver resection for metastatic colorectal carcinoma. *Ann Surg* 1992; **216**: 493-504; discussion 504-505

22 **Franco D**, Capussotti L, Smadja C, Bouzari H, Meakins J, Kemeny F, Grange D, Dellepiane M. Resection of hepatocellular carcinomas. Results in 72 European patients with cirrhosis. *Gastroenterology* 1990; **98**: 733-738

23 **Stone HH**, Long WD, Smith RB 3rd, Haynes CD. Physiologic considerations in major hepatic resections. *Am J Surg* 1969; **117**: 78-84

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LIVER CANCER

Efficacy of ultrasonography and alpha-fetoprotein on early detection of hepatocellular carcinoma

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Abstract

AIM: To evaluate the effectiveness of ultrasonographic screening for early detection of hepatocellular carcinoma (HCC).

METHODS: The data of 14 968 patients who had ultrasonography (US) for chronic liver diseases were collected into a database program from June 1995 to June 2005. The risk factors for HCC were also studied. A total of 6089 patients who had repeated US were enrolled, 264 patients were diagnosed with HCC during follow-up (mean, 39 mo).

RESULTS: The detection rate of small HCC (≤ 3 cm in diameter) was 67.7%. The tumor size detected by screening at the intervals of 6 mo was significantly smaller than that at longer intervals. Only 29.3% of HCC patients had an elevated serum alpha fetoprotein (AFP) level above 400 ng/mL. The risk of HCC development during follow-up was higher in patients with liver cirrhosis (10.9%) and hepatitis C (9.0%) than in patients with chronic hepatitis (4.2%), hepatitis B (4.9%) and non-B, non-C hepatitis (NBNC, 3.9%).

CONCLUSION: US screening at a interval of 6 mo is beneficial to high-risk patients over 40 years old and the early detection of HCC prolongs survival.

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Key words: Hepatitis C; Hepatocarcinogenesis; Interferon; Retreatment

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks fifth in frequency of cancers worldwide[1-3]. As the prognosis of HCC is extremely poor and effective treatment for patients with advanced HCC has not yet been established, the early detection of HCC is important for its effective treatment. Chronic hepatitis B and C as well as cirrhosis, irrespective of their etiology, are recognized as the major factors increasing the risk of HCC[4-7]. Thus, HCC screening has been extended to include patients with chronic hepatitis B or C as those with cirrhosis[8,9]. The most important tumor marker for HCC is alpha-fetoprotein (AFP). The common method for screening high risk patients using AFP marker can detect more early tumors and prolong the survival of patients[10]. Although HCC screening test has become an accepted procedure among high risk populations, there are still some arguments about its effectiveness because there is no randomized controlled study showing a decrease in disease mortality[11,12]. In addition, the usefulness, frequency and cost-effectiveness of HCC screening may differ in different areas, which may reveal a different prevalence of hepatitis B or C. The aim of this study was to evaluate the usefulness of a screening system in early diagnosis of HCC and to assess the risk factors for HCC development in patients with chronic liver diseases.

MATERIALS AND METHODS

Patients

From June 1995 to June 2005, all data of patients undergone US for HCC or chronic liver diseases in our hospital were collected into a special database program. A total of 14 968 patients had 29 926 examinations during a period of 10 years. We excluded patients with a history of liver cancer or other diseases that might affect survival. In addition, patients with focal hepatic lesion detected at admission or within 3 mo after enrollment were also excluded. All patients were periodically followed up for occurrence of any liver disease. A total of 6089 patients enrolled in this study had US periodically for at least 1 year more.

To assess the risk factors for HCC development, the clinical parameters were collected from 1982 patients. We entered all detailed data into the database program. The age distribution was mostly between the fifth and seventh decades (data not shown). Seventy-seven percent of subjects were positive for hepatitis B surface antigen (HBsAg), 15.5% were positive for anti-hepatitis C virus

Table 1 Comparison of liver status at initial screening and detection of HCC

Liver status	Patients, *n* (%)	
	At initial screening	At detection of HCC
CH	63 (23.9)	9 (3.4)
LC	201 (76.1)	255 (96.6)
Child A	85 (42.2)	122 (47.8)
Child B	38 (19.0)	58 (22.7)
Child C	78 (14.9)	75 (26.1)

Table 2 Underlying liver diseases at initial US of patients with HCC, *n* (%)

Liver status	HBV (+)	HCV (+)	BC	NBNC	Total
CH	45 (17.3)	13 (4.9)	3 (1.1)	3 (0.6)	42 (23.5)
LC	145 (54.8)	37 (14.0)	2 (0.9)	17 (6.4)	201 (76.1)
Total	190 (72.1)	50 (18.9)	5 (2.0)	19 (7.0)	264 (100.0)

Table 3 Tumor size at diagnosis

Diameter (cm)	Patients, *n* (%)
≤ 2	106 (40.8)
> 2-3	70 (26.9)
> 3-5	57 (21.9)
> 5	27 (10.4)
Total	260 (100.0)

Table 4 Tumor size according to US interval

US interval (mo)	Patients, *n* (%)	Tumor size (cm)
≤ 6	124 (47.2)	2.7 ± 1.3[a]
≤ 3	26 (10.0)	3.4 ± 1.8
4-6	98 (37.2)	2.5 ± 1.1
> 6	140 (52.8)	3.4 ± 2.0
7-12	90 (34.0)	3.0 ± 2.1
> 12	50 (18.8)	4.4 ± 1.7
Total	264 (100.0)	3.1 ± 1.7

[a]$P < 0.01$ *vs* > 6-mo group.

Table 5 Median survival according to tumor size

Diameter	Patients, *n* (%)	Median survival (mo)
≤ 2	106 (40.8)	42
> 2-3	70 (26.9)	35
> 3-5	57 (21.9)	20
> 5	26 (10.4)	10
Total	260 (100.0)	31

Table 6 Serum AFP level at detection of HCC

AFP level (ng/mL)	Patients, *n* (%)
≤ 20	107 (41.1)
≥ 1-400	86 (29.6)
> 400	70 (29.3)

(anti-HCV), 0.5% were positive for both and 8.4% were negative for both.

All patients were prospectively monitored by measurement of serum AFP and US at the intervals of 3 mo or 12 mo according to the status of underlying liver disease. The mean follow-up time was 39 ± 28 mo. The duration of HCC development was calculated by the time interval between the date of initial US and diagnosis of HCC. When US showed a new focal lesion or the serum AFP was increased, additional investigations were performed, such as a repeated AFP test, contrast computed tomography, or magnetic resonance imaging. HCC was diagnosed by histology or typical imaging features and elevated serum AFP level (≥ 400 ng/mL).

Serum AFP level was measured by a commercial enzyme-linked immunosorbent assay kit (Abbott, North Chicago IL, USA). US was performed by internal physicians.

Statistical analysis

The data were analyzed using the chi square test, logistic regression, or Kaplan-Meier method. All statistical analyses were done with SAS program and SPSS for windows (7.5.1) (Kaplan-Meier method and Cox regression hazard model).

RESULTS

HCC was found in 264 patients by screening during follow-up, and the annual detection rate was 2.1%. At enrollment, cirrhosis was found in 201 patients (76.1%), chronic hepatitis was diagnosed in 63 patients (23.9%). However, most patients (96.6%) progressed to liver cirrhosis when HCC was detected (Table 1). Of the 264 HCC patients, 190 (72.1%) were associated with hepatitis B, 50 (18.9%) with hepatitis C, and 19 (7.0%) not associated hepatitis B or C (Table 2).

The mean diameter of tumor at diagnosis was 3.1 ± 1.7 cm, and the detection rate of small HCC (≤ 3 cm) was 67.7%. Of the 260 patients, 27 (10.4%) had a tumor larger than 5 cm in diameter (Table 3). The mean diameter of the detected tumors at the intervals of 6 mo was significantly smaller than that at longer intervals (2.7 cm *vs* 3.4 cm, $P < 0.01$). However, there was no difference in tumor size at different time intervals (Table 4). The median survival time was 31 mo (Table 5) and the smaller the tumor size the longer the survival time. Of the 263 HCC patients, 70 (29.3%) had an elevated serum AFP level above 400 ng/mL (Table 6). However, the serum AFP levels were below 20 ng/mL in 107 patients (41.1%).

The incidence of HCC was higher in patients with liver cirrhosis (10.9%) than in those with chronic hepatitis (4.2%) and HBsAg carriers (1.5%). No patient with fatty liver developed HCC. The incidence of HCC was higher in hepatitis C-related group (9.0%) than in hepatitis B- (4.9%) and NBNC-related group (3.9%). However, there was no significant difference between hepatitis C and B patients older than 40 years.

DISCUSSION

The prognosis of HCC is poor especially in Africa and China[13]. By contrast, the disease runs a more benign course in patients in low risk regions, although they have a mean survival time of only about 6 mo[14]. The role of chronic infection with HBV and HCV in the etiology of liver cancer is well established[15]. Some 360 million people are chronically infected and at risk of death due to cirrhosis and HCC. Five hundred thousand to 700 000 people die of HBV-related liver diseases each year[16]. More than 170 million people worldwide are chronically infected with HCV, which is responsible for more than 100 000 cases of liver cancer per year[17].

The usefulness, frequency, and cost-effectiveness of HCC screening may differ in different geographic areas or in different underlying liver disease populations because there may be differences in the incidence and growth characteristics[9,11]. Although US screening for HCC is still controversial, it is a generally accepted strategy in East Asian countries. As patients with chronic hepatitis or cirrhosis may develop HCC, emphasis has been placed on the early detection of HCC when it is small, asymptomatic and potentially curable, by screening patients at high risk[18]. Several trials in areas of high and low HCC incidence have demonstrated that screening can detect patients at early stage of the disease, increase the resection rate and prolong survival time. Randomized prospective studies in China on high risk patients showed that the survival rate of patients after HCC resection is 52.7% after three years[19,20].

Cirrhosis is recognized as the major risk factor for HCC, and the annual risk of developing HCC is between 1% and 6%[21-25]. In our study, the annual risk of HCC development in cirrhosis patients was 3.5%. Reported screening studies showed that 20%-56% of HCC patients have undiagnosed cirrhosis[26,27]. Screening has been extended to include patients with chronic viral hepatitis as well as those with cirrhosis. The overall annual detection rate of HCC in surveillance studies including chronic hepatitis varies from 0.8% to 4.1%[11,28-31]. In our study, the annual detection rate of chronic hepatitis was 1.19%.

If the sample size of the target population or the number of HCC cases is not large enough, it is difficult to evaluate the effectiveness of screening in a high-risk population with different status and etiology of liver diseases. Therefore, a large target population and a sufficient number of HCC cases can avoid sampling bias. In our study, the study subjects were enrolled from 29 926 patients who had US for HCC screening or for various chronic liver diseases. We enrolled 6089 patients who had US periodically for a period of at least 1 year by using a database program. Our study included a variety of liver diseases such as hepatitis B or C, fatty liver and liver cirrhosis-related hepatitis B and C, or non-B and C. Two hundred and sixty-four patients were found to have HCC during a mean 39-mo follow-up, and the annual detection rate was 2.0%.

As the sample size was large enough to allow estimation, we tried to evaluate the efficacy of HCC screening. Because our study was a clinic-based screening, the incidence of HCC was higher than that of Sherman *et al*[9]. In our study, 76.9% of patients with HCC had liver cirrhosis and 23.1% had chronic hepatitis. Furthermore, most of these cases progressed to liver cirrhosis before development of HCC. Although the incidence of HCC was higher in liver cirrhosis patients (10.7%) than in chronic hepatitis patients (4.0%), chronic hepatitis B or C might progress to cirrhosis and HCC, suggesting that surveillance is needed to include patients with chronic viral hepatitis over the age of 40 years in China.

Reported screening intervals vary from 3 to 12 mo. A 6-mo interval is generally accepted as a rational choice. However, some others prefer shorter intervals in high-risk patients[32,33]. In our study, the mean diameter of tumor size at diagnosis was 3.1 ± 1.7 cm and the detection rate of small HCC (< 3 cm) was 67.7%. However, there was no difference in tumor size between different time intervals (Table 4). Although a shorter screening interval can detect earlier HCC, it decreases cost-effectiveness.

Whether early detection of tumors can prolong survival time remains unclear[11]. In our study, the overall median survival of the screened group was 31 mo, suggesting that survival time correlates well with tumor size. Furthermore, survival time was remarkably improved when the detected tumor size was equal or smaller than 2 cm (Table 5). Although the resection rate was relatively low in our study, most tumors, especially those less than 3 cm in diameter, were amenable to non-surgical curative or effective therapy.

Only 70 of the 264 HCC patients (29.3%) had an elevated serum AFP level above 400 ng/mL. In addition, the serum AFP level was below 20 ng/mL in 41.1% of patients. Trials in China have shown that combination of US and AFP can achieve better results in screening HBsAg positive subjects[21,34].

The incidence of HCC was higher in liver cirrhosis patients (10.9%) than in chronic hepatitis patients (4.2%). None of the patients with fatty liver developed HCC. The incidence of HCC was higher in hepatitis C-related group (9.0%) than in hepatitis B- (4.9%) and NBNC-related groups (3.9%). However, the incidence was not significantly different between hepatitis C and B in the group over 40 years of age. As the risk of HCC is negligible in hepatitis B patients under the age of 30 years and in hepatitis C patients under the age of 40 years, the screening would be better restricted to patients over the age of 40 years.

In conclusion, US screening at the intervals of 6 mo is beneficial to high-risk patients over the age of 40 years and prolongs survival. According to risk factors, the necessity for a screening test and a proper interval should be reconsidered.

REFERENCES

1 **Tang ZY**. Hepatocellular carcinoma-cause, treatment and metastasis. *World J Gastroenterol* 2001; **7**: 445-454

2 **Bruix J**, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 2002; **35**: 519-524

3 **Okuda K.** Hepatocellular carcinoma. *J Hepatol* 2000; **32**: 225-237

4 **Yu MC**, Yuan JM. Environmental factors and risk for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S72-S78

5 **Velázquez RF,** Rodríguez M, Navascués CA, Linares A, Pérez R, Sotorríos NG, Martínez I, Rodrigo L. Prospective analysis of risk factors for hepatocellular carcinoma in patients with liver cirrhosis. *Hepatology* 2003; **37**: 520-527

6 **Ohata K**, Hamasaki K, Toriyama K, Matsumoto K, Saeki A, Yanagi K, Abiru S, Nakagawa Y, Shigeno M, Miyazoe S, Ichikawa T, Ishikawa H, Nakao K, Eguchi K. Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. *Cancer* 2003; **97**: 3036-3043

7 **Yano Y**, Yamashita F, Sumie S, Ando E, Fukumori K, Kiyama M, Oyama T, Kuroki S, Kato O, Yamamoto H, Tanaka M, Sata M. Clinical features of hepatocellular carcinoma seronegative for both HBsAg and anti-HCV antibody but positive for anti-HBc antibody in Japan. *Am J Gastroenterol* 2002; **97**: 156-161

8 **Villeneuve JP**, Desrochers M, Infante-Rivard C, Willems B, Raymond G, Bourcier M, Côté J, Richer G. A long-term follow-up study of asymptomatic hepatitis B surface antigen-positive carriers in Montreal. *Gastroenterology* 1994; **106**: 1000-1005

9 **Sherman M**, Peltekian KM, Lee C. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. *Hepatology* 1995; **22**: 432-438

10 **Lopez JB**. Recent developments in the first detection of hepatocellular carcinoma. *Clin Biochem Rev* 2005; **26**: 65-79

11 **Collier J**, Sherman M. Screening for hepatocellular carcinoma. *Hepatology* 1998; **27**: 273-278

12 **Di Bisceglie AM**, Carithers RL Jr, Gores GJ. Hepatocellular carcinoma. *Hepatology* 1998; **28**: 1161-1165

13 **Kew MC**, Geddes EW. Hepatocellular carcinoma in rural southern African blacks. *Medicine* (Baltimore) 1982; **61**: 98-108

14 **Kew MC**, Dos Santos HA, Sherlock S. Diagnosis of primary cancer of the liver. *Br Med J* 1971; **4**: 408-411

15 **Bosch FX**, Ribes J, Borràs J. Epidemiology of primary liver cancer. *Semin Liver Dis* 1999; **19**: 271-285

16 Website of the world health organization. Accessed 2 Febtuary 2005. Available from: URL: http://www.who.int/mediacentre/factsbeets/fs204/en/

17 **Poynard T**, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; **362**: 2095-2100

18 **Di Bisceglie AM**, Rustgi VK, Hoofnagle JH, Dusheiko GM, Lotze MT. NIH conference. Hepatocellular carcinoma. *Ann Intern Med* 1988; **108**: 390-401

19 **Yang B**, Zhang B, Xu Y, Wang W, Shen Y, Zhang A, Xu Z. Prospective study of early detection for primary liver cancer. *J Cancer Res Clin Oncol* 1997; **123**: 357-360

20 **Zhang B**, Yang B. Combined alpha fetoprotein testing and ultrasonography as a screening test for primary liver cancer. *J Med Screen* 1999; **6**: 108-110

21 **Kato Y**, Nakata K, Omagari K, Furukawa R, Kusumoto Y, Mori I, Tajima H, Tanioka H, Yano M, Nagataki S. Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. *Cancer* 1994; **74**: 2234-2238

22 **Ikeda K**, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, Kumada H, Kawanishi M. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993; **18**: 47-53

23 **Colombo M**, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, Donato MF, Piva A, Di Carlo V, Dioguardi N. Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991; **325**: 675-680

24 **Oka H**, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994; **19**: 61-66

25 **Pateron D**, Ganne N, Trinchet JC, Aurousseau MH, Mal F, Meicler C, Coderc E, Reboullet P, Beaugrand M. Prospective study of screening for hepatocellular carcinoma in Caucasian patients with cirrhosis. *J Hepatol* 1994; **20**: 65-71

26 **Liver Cancer Study Group of Japan.** Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. *Ann Surg* 1990; **211**: 277-287

27 **Zaman SN**, Johnson PJ, Williams R. Silent cirrhosis in patients with hepatocellular carcinoma. Implications for screening in high-incidence and low-incidence areas. *Cancer* 1990; **65**: 1607-1610

28 **Takano S**, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21**: 650-655

29 **Chiba T**, Matsuzaki Y, Abei M, Shoda J, Tanaka N, Osuga T, Aikawa T. The role of previous hepatitis B virus infection and heavy smoking in hepatitis C virus-related hepatocellular carcinoma. *Am J Gastroenterol* 1996; **91**: 1195-1203

30 **Curley SA**, Izzo F, Gallipoli A, de Bellis M, Cremona F, Parisi V. Identification and screening of 416 patients with chronic hepatitis at high risk to develop hepatocellular cancer. *Ann Surg* 1995; **222**: 375-80; discussion 380-3

31 **Tsukuma H**, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797-1801

32 **Chen DS**, Sung JL, Sheu JC, Lai MY, How SW, Hsu HC, Lee CS, Wei TC. Serum alpha-fetoprotein in the early stage of human hepatocellular carcinoma. *Gastroenterology* 1984; **86**: 1404-1409

33 **Sheu JC**, Sung JL, Chen DS, Yang PM, Lai MY, Lee CS, Hsu HC, Chuang CN, Yang PC, Wang TH. Growth rate of asymptomatic hepatocellular carcinoma and its clinical implications. *Gastroenterology* 1985; **89**: 259-266

34 **Tong MJ**, Blatt LM, Kao VW. Surveillance for hepatocellular carcinoma in patients with chronic viral hepatitis in the United States of America. *J Gastroenterol Hepatol* 2001; **16**: 553-559

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COLORECTAL CANCER

Expression of NOS and HIF-1α in human colorectal carcinoma and implication in tumor angiogenesis

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Abstract

AIM: To study CD34, CD105, inducible nitric oxide synthase (iNOS), endogenous nitric oxide synthase (eNOS), and hypoxia-inducible factor 1 (HIF-1) α expression in human colorectal carcinomas.

METHODS: The tissue microarrays (TMAs) were made up of 80 cases of colorectal carcinoma and 80 cases of non-neoplasm colorectal mucosa. The expression of CD34, CD105, NOS and HIF-1α was detected by immunohistochemistry (S-P).

RESULTS: iNOS and HIF-1α expression in colorectal carcinoma was significantly higher than in non-neoplasm colorectal mucosa (χ^2 = 43.166, P < 0.01; χ^2 = 10.4278, P < 0.01); eNOS expression in colorectal carcinoma was significantly lower than in non-neoplasm colorectal mucosa (χ^2 = 11.354, P < 0.01). The expression of iNOS correlated with differentiation (χ^2 = 18.141, P < 0.01), invasive depth (χ^2 = 4.748, P < 0.01), and Micro vessel density (MVD) (t = 2.327, P < 0.05). The expression of HIF-1α was correlated with infiltrating depth (χ^2 = 4.397, P < 0.05), Duke's staging (χ^2 = 4.255, P < 0.05), and MVD (t = 2.272, P < 0.05). No correlation was found in eNOS expression.

CONCLUSION: Over-expression of iNOS and HIF-1α in colorectal carcinoma is correlated with the biological character MVD.

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Key words: Nitric oxide synthase; HIF-1α; Colorectal carcinoma; Angiogenesis

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INTRODUCTION

Angiogenesis is an important step in the growth and metastasis of solid malignant tumors. To adapt to hypoxia, angiogenesis is essential for tumor growth. Tumor must over-express genes that encode angiogenic factors to induce new blood vessels[1]. A number of angiogenic factors have been found, and there is interrelation among them, which has been widely researched. Nitric oxide synthase (NOS) and hypoxia-inducible factor 1 (HIF-1) α are two of these factors which may be correlated with angiogenesis. To screen out angiogenic factors is time-consuming and expensive. Tissue microarray (TMA) brings to pathologists a completely new method to solve this problem. TMA has many excellent features such as small volume, high information content, and assembling differently according to different needs. A number of results can be obtained through one experiment[2], which reduces research time and improves efficiency greatly. Moreover, the most important advantage is a decrease of experimental error, because TMA can make all samples under the same experimental condition. To study NOS and HIF-1α as angiogenic factors, we used immunohistochemistry to detect the expression of CD34 and CD105 and the expression of inducible NOS (iNOS), endogenous NOS(eNOS), and HIF-1α in human colorectal carcinomas. TMAs consisting of 80 colorectal carcinoma and 80 non-neoplasm colorectal mucosa were employed to accomplish these studies.

MATERIALS AND METHODS

Patients

Eighty specimens were collected from 80 patients with colorectal carcinoma (median age 62.2 ± 5.9 years, range 29-88 years) who were operated in Qingdao Municipal Hospital (Shandong, China) from Oct 2003 to Mar 2004. Carcinoma and non-neoplasm colorectal mucosa (located at least 8 cm away from the margins of cancers) were taken from each sample. There were 6 cases of well-differ-

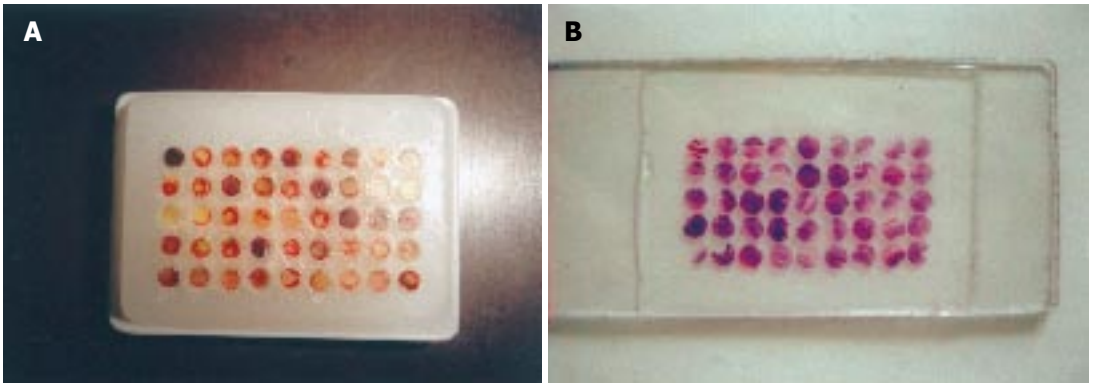

Figure 1 Tissue microarray. **A**: Block; **B**: Section (HE).

entiated adenocarcinoma, 59 cases of moderately differentiated adenocarcinoma and 15 cases of poorly differentiated adenocarcinoma. Among them, 30 cases had regional lymph node metastasis and 12 cases had distant metastasis. None of the patients received any chemotherapy or radiation therapy before surgical operation. The pathological diagnosis was made on the basis of the size, infiltrating depth, histological grade of the carcinoma, and lymph node and distant metastasis. The grading standard was in accordance with that established in the book *Practical and Surgical Pathology*[3].

Tissue microarray

HE staining sections of all samples were observed firstly, and typical lesion positions were marked. Then the corresponding positions to these lesions were marked on paraffin wax. The empty TMA paraffin wax (45 mm × 28 mm × 7 mm) was holed through Tissue Arrayer (each diameter was 2 mm), and the tissues taken from marked positions were put in these holes tidily (each diameter was also 2 mm). The process was repeated for many times and the TMA paraffin wax was ready (Figure 1A). A different kind of tissue was put in it as a marker, which was kidney tissue in this experiment. Each TMA paraffin wax was sliced into 4 μm thick sections.

Immunohistochemistry

The expression of CD34, CD105, NOS and HIF-1α was stained by immunohistochemistry (S-P). The reagent kits were purchased from Fuzhou Maxim Co. Micro vessel density (MVD) was counted with different vessel markers CD34 and CD105 respectively. CD34 and CD105 antibodies were supplied from Neomarker Company, and working consistence was 0.2 mg/L, and 0.4 mg/L. iNOS and eNOS multi-clonal antibody was purchased from Neomarker Company; working consistence was 20 mg/L, and 20 mg/L, respectively; multi-clonal HIF-1α antibody was from Santa Cruz Company; working consistence was 0.8 mg/L. Homogenous animal IgG that contained the same protein quantity was used instead of first antibody in negative control.

NOS and HIF-1α staining was classified into two groups. Positive group includes: +++, most carcinoma cells were stained with a very strong intensity, and distributed in clusters; ++, a large number of carcinoma cells were stained with a moderate intensity, and distributed in clusters occasionally; +, a few carcinoma cells were stained with a slight intensity. Negative group is defined as: -, no staining of carcinoma cells.

Counting of MVD in carcinoma tissues was in accordance with Weidner's standards with a minor modification. The sections were searched for the hot spots rich in vessels, which were located in or near the area of tumor tissues under a low power microscope (100 ×). MVD was counted under a high power (400 ×) microscope according to the standards that any stained endothelial cell or cells were identified as an independent vessel. These vessels must be clearly separated from each other. However, apparent vasa or vasa with red blood cells could be regarded as vessels. Three different HP vision fields were chosen on each of the sections. Stained vessels were counted by two pathologists under multi-ocular microscope simultaneously. The results were averaged as the relative value of the amount of vessels per unit area.

Statistical analysis

The difference of MVD between CD105 and CD34 staining was analyzed with paired sample *t*-test. The relation between MVD and expression of NOS and HIF-1α was analyzed with *t*-test. Pearson-chi-square test was used for statistical analysis of the relation between clinical-pathological parameters and expression of NOS and HIF-1α.

RESULTS

Tissue microarray evaluation

Every TMA section consisted of 36-46 samples, which were arrayed orderly. The shape of these samples was rounded or elliptoid, and a few samples were crimpled or lost (Figure 1B). After TMA wax was sliced consecutively, one section was picked out for HE staining to make sure that the tissue still kept its histological feature. In fact, almost all samples could keep their histological feature in TMA wax which total depth was about 3 mm. Adjacent sections that contained all samples were selected. After antigen retrieval and IHC staining, 1-2 samples fell off from the sections. Therefore, the number of available samples was 78, and the availability was 97.5%.

Expression of CD34/CD105 and MVD

CD105 was mainly expressed in cytoplasm and plasmalemma of newborn endothelial cells. These positive cells were

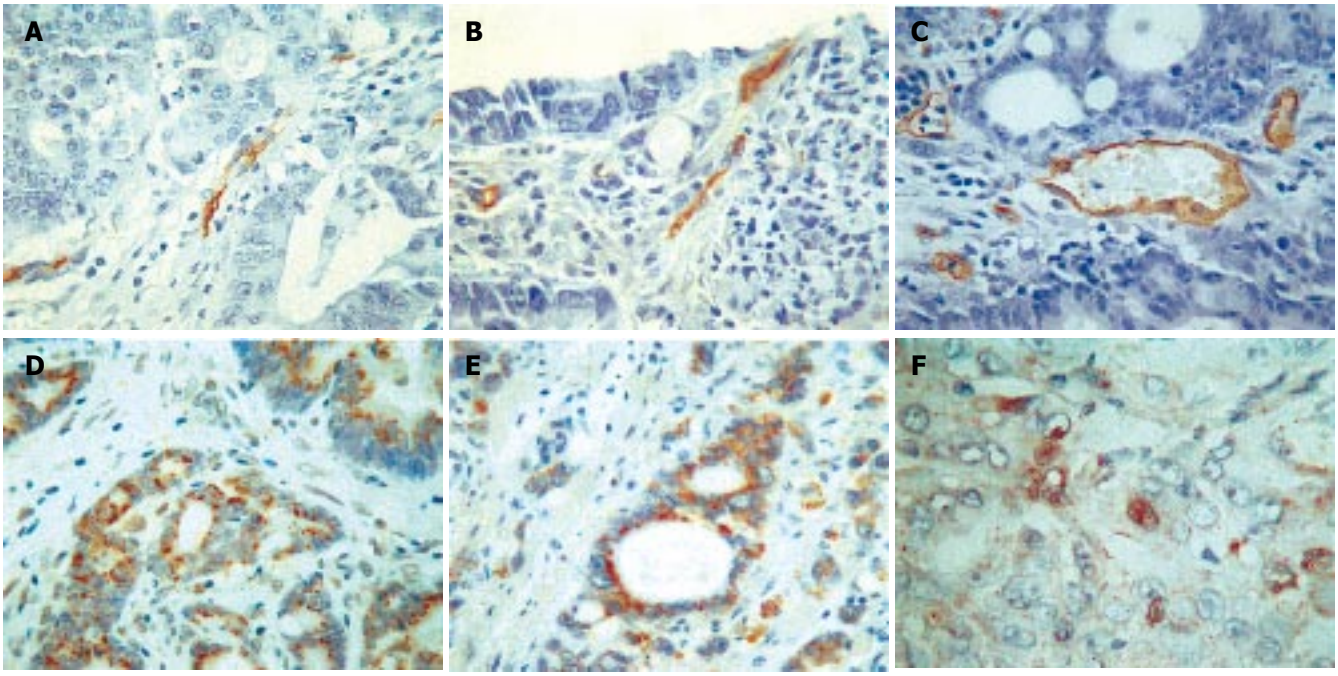

Figure 2 CD105, CD34, iNOS, eNOS and HIF-1α expressions in colorectal carcinoma (SP × 400). **A**: CD105 in vessels; **B**: CD105 not in vessels; **C**: CD34; **D**: iNOS; **E**: eNOS; **F**: HIF-1α.

shown as one cell or a group of cells, and most of them ranged in a line or an irregular goblet while a few of them formed lumen. CD105 was weakly expressed or absent in native blood vessels with thick walls or large lumina (Figure 2A). The vessels with positive CD34 staining formed lines or had obvious lumina. Endothelial cells of many large vessels with thick walls and smooth muscles showed positive staining, which was distinct from the sections of CD105 staining (Figure 2B and C). *t*-test showed that MVD by CD34 staining was significantly different from that by CD105 staining (27.5 ± 10.3 *vs* 14.0 ± 5.8, $t = 4.782$, $P < 0.01$).

Expression of iNOS and eNOS

The positive rate of iNOS was 78.2% (61/78) in colorectal carcinoma, and 25.6% (20/78) in non-neoplasm mucosa of colorectal tissue. The difference between them was significant ($\chi^2 = 43.166$, $P < 0.01$). The positive granules of iNOS were mainly located in cytoplasm diffusely (Figure 2D). The positive rate of iNOS decreased with the descending of differentiation grade. Most ring carcinoma showed negative expression. The expression of eNOS showed positive or weak positive in non-neoplasm mucosa of colorectal tissue. The positive rate was 60.3% (47/78). The positive granules also located diffusely in cytoplasm, and some expressed in cell nucleus (Figure 2E). The expression of eNOS in colorectal carcinoma was 33.3% (26/78), which was statistically different from that in non-neoplasm mucosa of colorectal tissue ($\chi^2 = 11.354$, $P < 0.01$). The positive rate of eNOS also decreased with the descending of differentiation grade. (Table 1)

Expression of HIF-1α

The positive rate of HIF-1α was 69.2% (54/78) in colorectal carcinoma, and 43.6% (34/78) in non-neoplasm mucosa of colorectal tissue ($\chi^2 = 10.4278$, $P < 0.01$). The positive granules mainly located in cell nucleus and cytoplasm (Figure 2F). A small number of HIF-1α granules were expressed in inflammatory cells in the stroma of tumor.

Table 1 Relation between expression of iNOS, eNOS, HIF-1α and clinical pathology, *n* (%)

Pathology	*n*	iNOS	eNOS	HIF-1α
Histological grade				
Well differentiation	6	5 (83.3)[b]	3 (50)	2 (33.3)
Moderate differentiation	58	51 (87.9)	21 (36.2)	43 (74.1)
Poor differentiation	14	5 (35.7)	2 (14.3)	9 (64.3)
Infiltrating depth				
Serous membrane (-)	9	4 (44.4)[d]	2 (22.2)	3 (33.3)[a]
Serous membrane (+)	69	57 (82.6)	24 (34.8)	51 (73.9)
Lymph node metastasis				
(+)	29	24 (82.8)	11 (37.9)	23 (79.3)
(-)	49	36 (77.9)	15 (33.3)	31 (63.3)
Distant metastasis				
(+)	12	11 (91.7)	5 (41.7)	11 (91.7)
(-)	66	50 (75.8)	21 (31.8)	43 (65.2)
Duke's stage				
A, B	45	35 (77.8)	18 (40.0)	27 (60)[c]
C, D	33	26 (78.8)	8 (24.2)	27 (81.8)

[a]: $P < 0.05$ between serous membrane positive group and negative group;
[c]: $P < 0.05$ between Duke's stage A&B group and C&D group;
[b]: $P < 0.01$ between different histological grade groups;
[d]: $P < 0.01$ between serous membrane positive group and negative group.

Relation between NOS/HIF-1α and MVD

Pearson-Chi-square test showed that the expression of iNOS was correlated with differentiation ($\chi^2 = 18.141$, $P < 0.01$), and infiltrating depth ($\chi^2 = 4.748$, $P < 0.01$). The

expression of eNOS had no significant difference in different groups. However, the expression of HIF-1α was correlated with infiltrating depth ($\chi^2 = 4.397$, $P < 0.05$), and Duke's staging ($\chi^2 = 4.255$, $P < 0.05$). MVD of iNOS positive group was significantly higher than that of the negative group (14.3 ± 6.1 *vs* 10.6 ± 4.0, $t = 2.327$, $P < 0.05$); HIF-1α staining showed the same result (15.2 ± 5.4 *vs* 11.5 ± 4.3, $t = 2.272$, $P < 0.05$), while the MVD between the two groups of eNOS staining had no significant difference (13.8 ± 5.4 *vs* 11.9 ± 4.7, $t = 1.523$, $P > 0.05$).

DISCUSSION

Angiogenesis refers to the formation of new blood vessels from native blood vessels, which is very important to the growth and invasion of malignant solid tumors[4]. In 1971, Folkman firstly put forward the viewpoint that the growth of tumor depends on angiogenesis[5]. In tumor microenvironment angiogenesis needs many steps, which is a continuous process. The characteristics of micro vessels in tumors are different from that developed under normal physiological conditions. In fact, MVD has been extensively investigated and was found to be a useful prognostic marker in many types of cancers[6].

At present, most researchers use immunohistochemistry (IHC) to study new blood vessel in tumors. Therefore it is necessary to select an appropriate marker of endothelial cells[7]. Previous reports have demonstrated that MVD-CD34 is a better prognostic marker than MVD assessed by many other endothelial markers, such as von Willebrand factor (vWF), CD31. However, CD34 is a pan-endothelial cell marker, thus it is difficult to distinct newborn vessels from native vessels, and it may not be able to reflect the exact angiogenesis activity in a tumor. According to some previous reports, CD105 is an endothelial marker that appears to react only with the endothelial cells in the newly formed vessels, and in particular, the immature tumor blood vessels[8]. Based on our study, we suggest that CD105 has a higher rate of positivity and stronger reactivity on the endothelial cells of new blood vessels[9]. Therefore CD105 is a better marker for the endothelial cells of new blood vessels. In addition, CD105 monoclonal antibody in the experiment was suitable for paraffin sections. It overcame the limit that usual antibody is only used in frozen sections. Because frozen sections were more difficult to observe than paraffin sections, it led to the inconvenience for MVD counting under microscope. Using this antibody, we saved time and materials, and obtained better staining effect. Moreover, the result of the experiment was more reliable and accurate.

NOS includes eNOS and iNOS, which have different functions. eNOS is involved in many physiological functions of neural system, such as regulating the angioectasis and smooth muscle hyperplasia. iNOS expression in human diseases has long been investigated. NO production by iNOS in tumor infiltrating macrophages may be part of their anti-tumoral cytotoxic potential[10]. On the other hand, the expression of iNOS in endothelial cells of tumor vessels and in cancer cells itself supports the assumption that the cancer uses this form to regulate the tumor vascularisation and blood flow. eNOS exists in normal condition and only produce a slight number of NO, while iNOS is induced by activated cells to produce more NO which can promote the construction of tumor vessels. NO has been found to be a mediator of angiogenesis and blood flow[11,12].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional factor composed of two basic-helix-loophelix (bHLH)-PAS α and β subunits. HIF-1α is the unique, O_2-regulated subunit that determines HIF-1 activity[13]. A series of genes and proteins that may increase the survival of tumor cells under hypoxia conditions, including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), iNOS, platelet-derived endothelial growth factor (PDEGF), glucose transporter 1, lactate dehydrogenase, erythropoietin and nitric oxide synthase gene, are regulated by HIF-1α. Thus, HIF-1α may play important roles in tumor progression. Furthermore, HIF-1α is a critical factor of transcription in the pathway of vascular growth signals. It can activate many genes of angiogenic factors, which can lead to angiogenesis. Most studies about the effects of HIF-1α on the neovascularization in malignancies have been done *in vitro* or in animal models. It has been confirmed that in many cancer cell lines, hypoxia can induce the expression of some proangiogenic factors, including VEGF, IGF, and PDEGF, through HIF-1α-dependent manner. It has also been proven through the method of gene knockout that the loss of HIF-1α may significantly suppress the growth of tumors including glioblastoma and malignant teratoma and most importantly, it may decrease the neovascularization of those malignancies. This provides a new approach for the treatment of tumor[14].

Tissue chip is also called tissue microarray, which is a special biological chip invented by Kononen in 1998[15]. Its principle is to remove hundreds of minute tissues from different primary blocks and place them into one arrayed block with high density. Tissue microarray is suitable for all analyses *in situ* such as antibody staining, immunohistochemistry, fluorescence *in situ* hybridization, Western blot. Thus we can investigate the different expression of tissues either at DNA and RNA level or protein level. Tissue microarray is characterized with small bulk and high-throughput, which can produce many results through one experiment[2,16]. It can decrease experiment errors, greatly improve the efficiency of histopathological study, save experimental materials and reagents, and at the same time make the results more reliable and comparable[17-19]. Bubendorf *et al*[20] made a large amount of tissue microarrays of tumor, in which ten blocks had 4788 samples from 130 kinds of tumor. These microarrays can be used to analyse the difference of newly found genes between normal tissues and tumor, from which we can acquire reliable results. By using the same method, Fernebro *et al*[21] also found that Ki67 positive cell index of rectal carcinoma was 0.5-1.0 (average 0.85) by the method of microarray, being not significantly different from that of routine examination method (0.54-1.0, average 0.81); moreover, both methods had the same result on p53 (average 75%). These indicate that we could get reliable and appropriate results with tissue microarray for IHC staining. We have made tissue microarrays consisting of 80 cases of colorectal carcinoma, each of which has 40-50

samples, arranging orderly. Its shape is round or oval, with few crimples and it does not fall off easily. By using tissue microarrays, it is feasible to examine clinical tissue samples on large-scale and with high efficiency, which is rapid, convenient, economical and precise.

REFERENCES

1 **Fox SB**, Gatter KC, Leek RD, Harris AL, Chew KL, Mayall BH, Moore DH 2nd. More about: Tumor angiogenesis as a prognostic assay for invasive ductal breast carcinoma. *J Natl Cancer Inst* 2000; **92**: 161-162

2 **Zhou XG**, Zhang JS, Zhang XP. Tissue microarray. *Zhonghua Binglixue Zazhi* 2002; **31**: 70-72

3 **Chen ZN**. Practical and Surgical Pathology. 1st ed. Shanghai: Shanghai Medical University Press,1997

4 **Rosen LS**. Angiogenesis inhibition in solid tumors. *Cancer J* 2001; **7** Suppl 3: S120-S128

5 **Folkman J**. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; **285**: 1182-1186

6 **Newman PJ**, Berndt MC, Gorski J, White GC 2nd, Lyman S, Paddock C, Muller WA. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* 1990; **247**: 1219-1222

7 **Emoto M**, Iwasaki H, Mimura K, Kawarabayashi T, Kikuchi M. Differences in the angiogenesis of benign and malignant ovarian tumors, demonstrated by analyses of color Doppler ultrasound, immunohistochemistry, and microvessel density. *Cancer* 1997; **80**: 899-907

8 **Schimming R**, Marmé D. Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity. *Head Neck* 2002; **24**: 151-156

9 **Stacker SA**, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K, Achen MG. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem* 1999; **274**: 32127-32136

10 **Jenkins DC**, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. *Proc Natl Acad Sci USA* 1995; **92**: 4392-4396

11 **Papapetropoulos A**, García-Cardeña G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; **100**: 3131-3139

12 **Dulak J**, Józkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wójtowicz A, Szuba A, Cooke JP. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000; **20**: 659-666

13 **Sutherland RM**. Tumor hypoxia and gene expression--implications for malignant progression and therapy. *Acta Oncol* 1998; **37**: 567-574

14 **Semenza GL**. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* 2002; **8**: S62-S67

15 **Kononen J**, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847

16 **Yang J**, Wang KM, Su BS. Appliance of tissue microarray for in situ hybridization. *Linchuang Yu Shiyan Binglixue Zazhi* 2002; **18**: 344

17 **Gulmann C**, Loring P, O'Grady A, Kay E. Miniature tissue microarrays for HercepTest standardisation and analysis. *J Clin Pathol* 2004; **57**: 1229-1231

18 **Gancberg D**, Di Leo A, Rouas G, Järvinen T, Verhest A, Isola J, Piccart MJ, Larsimont D. Reliability of the tissue microarray based FISH for evaluation of the HER-2 oncogene in breast carcinoma. *J Clin Pathol* 2002; **55**: 315-317

19 **Pacifico MD**, Grover R, Richman P, Daley F, Wilson GD. Validation of tissue microarray for the immunohistochemical profiling of melanoma. *Melanoma Res* 2004; **14**: 39-42

20 **Bubendorf L**, Nocito A, Moch H, Sauter G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. *J Pathol* 2001; **195**: 72-79

21 **Fernebro E**, Dictor M, Bendahl PO, Fernö M, Nilbert M. Evaluation of the tissue microarray technique for immunohistochemical analysis in rectal cancer. *Arch Pathol Lab Med* 2002; **126**: 702-705

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Mental and physical symptoms associated with lower social support for patients with hepatitis C

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Abstract

AIM: To systematically examine the impact of the hepatitis C virus (HCV) diagnosis on patients' level of social support in a large-scale study.

METHODS: Patients evaluated and treated for HCV in a tertiary referral center were enrolled in a cross-sectional study. Demographic data, functional and emotional status as measured by the Hospital Anxiety and Depression Scale (HAD) and the Sickness Impact Profile (SIP), severity of liver disease, mode of acquisition, and physical and psychiatric comorbidities were collected from patients or abstracted from the medical record. All participants completed a semi-structured interview, addressing questions of social support.

RESULTS: A total of 342 patients (mean age 45.2 years; 37% women) were enrolled. Ninety-two (27%) patients described lower levels of support by family and friends. Nearly half of the participants (45%) noted the loss of at least one relationship due to the disease. Fears related to transmitting the disease (25%) were common and often associated with ignorance or even discrimination by others (19%). Nearly one fifth of the patients did not share information about their disease with others to avoid being stigmatized. Lower levels of social support were significantly associated with living alone, being unemployed, being excluded from antiviral therapy, having psychiatric comorbidities, contracting HCV through intravenous drug use, having high levels of anxiety and depression as measured by the HAD and negative mood state as measured by the SIP. Patients reporting lower levels of social support also noted more physical symptoms as measured by the SIP.

CONCLUSION: Patients with hepatitis C often face significant social problems, ranging from social isolation to familial stress. The most common concerns reflect a limited insight of patients and their relatives and friends about the disease, the risk factors for its spread, and about potential consequences. Our data suggest that educational interventions targeting support persons and the stressors identified in our findings may lessen or alleviate the social strains patients with hepatitis C experience.

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Key words: Hepatitis C; Social support; Depression; Anxiety; Quality of life; Stigmatization

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INTRODUCTION

Hepatitis C virus (HCV) infection, a common and slowly progressing liver disease, negatively affects quality of life. Most studies addressing the important impact of HCV focus on patients undergoing antiviral therapy[1-6]. However, even HCV-infected individuals outside of treatment have lower quality of life scores compared to uninfected controls[7-9]. Negative social interactions may play a unique role as a determinant of quality of life in patients with HCV, as they often face stigmatization in their professional and private environment[10-12]. Patients living with AIDS and stigmatizing diseases such as tuberculosis and leprosy

experience a deterioration or even complete collapse of their social support network[13-15]. Despite recent evidence of negative stereotyping in patients with HCV, few studies have focused on the effect of the disease on the patients' interactions with family, friends or other important groups[16]. Relatively little is known about possible correlations between social support and demographic and psychosocial or physical characteristics, or of the specific difficulties that patients experience in their lives[17]. In this analysis we hypothesize that poor social support is associated with emotional and physical difficulties in patients with HCV both in and outside of treatment. Using mixed quantitative and qualitative methods, we propose to characterize the nature and the causes of social problems as an important first step in alerting healthcare providers of the major stressors these patients face.

MATERIALS AND METHODS

Patients

The schedules of all hepatologists practicing at a large Midwestern teaching hospital were reviewed daily between October 1998 and May 2003 for patients meeting the study inclusion criteria. Patients younger than 18 years of age, prisoners, those unable to communicate verbally, or provide informed consent were excluded from the study. No exclusions for concurrent liver disease (hepatitis A or B) or HIV status were made. Patients with a confirmed diagnosis of HCV were invited to participate on the day of their clinic visit.

Data collection

Once informed consent was obtained, participants were asked to provide demographic information, to undergo an extensive interview addressing psychosocial and health-orientated questions (see Appendix), and to complete health status and psychosocial survey measures. The interview was designed to range in length from 30 min to two hours. Each interview was recorded with a hand-held tape recorder and transcribed verbatim. After the interview, patients provided demographic information (age, gender, race, marital status, education, population of home town), and completed the Hospital Anxiety and Depression Scale (HAD), a self-assessment scale for mood disturbances in a non-psychiatric patient population[18], and the Sickness Impact Profile (SIP), a behaviorally based health status measure including sub-scales for physical symptoms (Body Care and Movement, Ambulation, Mobility), psychosocial difficulties (Emotional Balance, Social Interaction, Alertness Behavior, physical ability to Communicate), as well as physical and psychosocial summary scores[19].

A physician abstracted from the electronic medical record patients' psychiatric diagnoses, Charlson Comorbidity Index[20], Child-Pugh score, dates of treatment for HCV infection (before, during, after interview), substance abuse, mode of transmission (drug use, blood transfusion/needle stick, tattoo/other/unknown), and response to treatment (durable responder, nonresponder/disease recurrence, naïve). Patients who were naïve either self-excluded from treatment or were excluded based on physical comorbidities, psychiatric disease, or substance use. Based on clinical records from the attending hepatologist, no patient was determined to remain naïve based on a lack of social support.

Coding

For the construction of our codebook[10,21] we used the quasi-statistical method as outlined by Miller and Crabtree[22]. We also employed the qualitative computer software program Atlas.ti (version 4.2, Berlin, Germany) to aid in the management and analysis of data. Twenty trained interviewers, each with over one year of experience in interviewing, read 50 out of 150 randomly selected interviews and ranked the top ten themes in terms of overall frequency and importance. The primary investigator and two trained coders then synthesized this list to create the master codebook which included the central themes related to social support, stigmatization, and quality of life. Using this codebook, each transcript was then coded by two independent coders, one who had undergone a minimum of three weeks formal training and a master coder with at least one-year experience. The initial coding sheets of each coder were converted into a spreadsheet to determine a final kappa score that assessed the agreement between the evaluators. The coders then met and processed the codes for each case until 100% agreement was achieved between them. From this a master code databank was developed which was then used for the statistical calculations.

For this analysis, the chief code was for social support, noting where patients described a dominantly negative environment (coded as 'unsupportive') versus all others (coded as 'supportive'). We defined social support as involving sexual partners, family members, and friends. An 'unsupportive' social environment was defined as dominantly negative (e.g., 'They have basically excluded me; so I'm all alone now,' 'Relations with my husband are just not good any more.') Conversely, a 'supportive' social environment was defined as either positive or mixed (*e.g.,* 'They are always there for me,' 'My in-laws won't have me in the house, but my wife and kids are okay with it.') When the interview was too conflicted or when too few details were provided, the code of 'cannot judge social situation' was used. These interviews were then excluded from the final analysis. The ordinal code of social support had an inter-coder reliability kappa score of 0.72, or what Landis and Kock label as a 'substantial' agreement between coders[23].

Two coders then conducted a close textual analysis of each discussion of social support present in the interviews. For the purpose of this analysis we concentrated on two main domains: (1) the negative impact of HCV on social support, and (2) the reasons for this negative impact.

Statistical analysis

All analyses were performed using SPSS version 12.0 (SPSS, Inc. Chicago, Illinois, USA). We first summarized the demographics and clinical characteristics of the patient sample, excluding anyone with missing data on social support. We then examined demographic, psychosocial,

and clinical characteristics of the patient sample, both overall and by level of social support, treating the level of social support as binary ('supportive' versus 'unsupportive'). We tested for differences between groups using ANOVA for the means of continuous variables (age, Child-Pugh score, HAD, SIP) and Chi-Square and Fisher's Exact for categorical variables, where appropriate. We then examined factors associated with social support. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 499 patients were approached, with 96 declining to participate, largely due to time constraints. Of the 403 patients successfully entered into the study, 61 were excluded due to either a technological failure in recording the interview (n = 12) or an inability to judge the social support code (n = 49). This left 342 patients in the total sample with a code for social support. This sample was (Table 1) predominantly male, white, and had acquired the disease mostly through intravenous drug use. Two hundred and fifty patients (73%) were categorized as having a 'supportive' and 92 (27%) as having an 'unsupportive' environment. Of all the patients in the study only 4 (3 with hepatitis B and 1 with HIV) had another chronic, sexually transmittable disease.

Table 1 Demographic and clinical characteristics of the study population

Patient characteristics	*n* (%) of total study population
Demographic characteristics	
Mean age in years (SD)	45.2 (9.2)
Women	128 (37.4)
Racial/ethnic background	
White	310 (90.6)
Black	19 (5.6)
Other[1]	13 (3.8)
Marital status	
Living with partner	212 (62.0)
Living alone	130 (38.0)
Education	
K-12	137 (40.1)
College	205 (59.5)
Employment prior to diagnosis	
Employed	296 (90.0)
Unemployed	33 (10.0)
Current employment	
Employed	217 (65.0)
Unemployed	117 (35.0)
Population of hometown	
Rural	220 (64.3)
Urban	122 (35.7)
Interview to treatment status	
Before	32 (9.4)
During	93 (27.2)
After	94 (27.5)
Naïve	123 (36.0)
Clinical characteristics	
Current substance use	
Yes	24 (7.0)
Total psychiatric diagnosis	
0	251 (73.6)
1	74 (21.7)
2	14 (4.1)
3-5	2 (0.6)
Advanced liver disease (cirrhosis)	81 (23.8)
HCV due to IV drugs	177 (62.3)
HCV due to other methods of transmission	107 (37.7)
Mean Child-Pugh score (SD)	5.46 (1.2)

SD: Standard Deviation; HCV: Hepatitis C virus; IV: Intravenous. [1]Other ethnicity includes Hispanic, Native American, Asian and Pacific Islander.

Bivariate analysis

Considering the cross-sectional design, participants were interviewed at different points during the evaluation of and/or treatment for HCV (pretreatment, treatment, post-treatment, naïve). Naïve patients (typically excluded from treatment due to psychiatric illness or substance use) reported more negative social experiences compared to patients in the pretreatment, treatment, and post-treatment groups (Table 2). Marital status (living without partner) and employment status (unemployed) were significantly associated with level of social support, while all other demographic and disease-related variables did not correlate. Psychiatric comorbidity and prior substance abuse as the mode of acquisition for HCV were associated with lower levels of support. The category of unsupportive social support also correlated with greater physical symptoms as measured by the physical summary scores of the SIP and all physical sub-scales (Body Care and Movement, Mobility, Ambulation). Similarly, lower support was associated with the psychosocial summary scores and all psychosocial sub-scales of the SIP (Emotional Balance, Social Interaction, Alertness, Communication), as well as higher scores for the depression and anxiety scales of the HAD (Table 2).

Thematic analysis

Two domains emerged as patients discussed their problems. First, they described the negative impact of the infection on their social support; second, they gave potential reasons for negative changes.

Negative impact of HCV on social support

Deterioration of relationships: One hundred and fifty-five patients (45% of the total sample) noted that having HCV had resulted in the breakdown or loss of at least one relationship. The deterioration of their social network affected sexual relationships (59 patients, 17% of the total sample) and interactions with family members (56 participants, 16% of the total sample): "I am estranged from my family. My wife and I divorced a year and a half ago. I didn't understand what was going on with me. One element of this disease is depression and an inability to think coherently sometimes. Being irrational, it's hard on relationships." Forty individuals (12% of the total sample) noted that they had experienced the loss of at least one friend.

Twenty-nine patients (8% of the total sample) mentioned multiple sources of loss: "Basically I do not have any good relationships with anyone." For these patients, the HCV diagnosis led to what they described

Table 2 Characteristics of groups categorized by social support

	Social support group		*P* value
	Supportive environment (*n* = 250)	Unsupportive environment (*n* = 92)	
Demographic characteristics			
Mean age in years (SD)	45.33 (9.82)	44.88 (7.52)	0.49
Women	91 (71.1)	37 (29.0)	0.52
Racial/ethnic background			0.09
White	229 (73.9)	81 (26.1)	
Black	15 (79.0)	4 (21.0)	
Other†	6 (46.2)	7 (53.8)	
Marital status			< 0.001[1]
Living with partner	171 (80.7)	41 (19.3)	
Living alone	79 (60.8)	51 (39.2)	
Education			0.59
K-12	98 (71.5)	39 (28.5)	
College	152 (74.1)	53 (26.0)	
Employment prior to diagnosis			0.63
Employed	218 (73.6)	78 (26.4)	
Unemployed	23 (70.0)	10 (30.0)	
Current employment			0.01[1]
Employed	169 (78.0)	48 (22.0)	
Unemployed	75 (64.1)	42 (36.0)	
Population of hometown			0.29
Rural	165 (75.0)	55 (25.0)	
Urban	85 (70.0)	37 (30.0)	
Interview to treatment status			0.03[1]
Before	23 (72.0)	9 (28.0)	
During	72 (77.4)	21 (23.0)	
After	76 (81.0)	18 (19.1)	
Naïve	79 (64.2)	44 (36.0)[1]	
Clinical characteristics			
Current substance use			0.8
Yes	17 (71.0)	7 (29.0)	
Total psychiatric diagnosis			
0	197 (78.5)	54 (22.0)	< 0.001[1]
1	42 (57.0)	32 (43.2)	
2	10 (71.4)	4 (29.0)	
3-5	0 (0.0)	2 (100.0)	
Advanced liver disease (cirrhosis)	63 (78.0)	18 (22.0)	0.27
HCV due to IV drugs	117 (66.0)	60 (34.0)	0.002[1]
HCV due to other methods of transmission	89 (83.2)	18 (17.0)	
Mean Child-Pugh score (SD)			0.68
Normal	234 (69.0)	85 (25.0)	
Elevated	15 (4.4)	7 (2.1)	
Mean HAD summary scores (SD)			
Depression score	4.04 (3.47)	6.95 (4.48)	< 0.001[1]
Anxiety score	6.57 (3.86)	8.95 (4.40)	< 0.001[1]
Mean SIP summary scores (SD)			
Physical score	4.64 (7.03)	10.4 (12.17)	< 0.001[1]
Psychosocial score	10.81 (13.67)	22.9 (18.82)	< 0.001[1]
Mean SIP subscale scores (SD)			
Social interaction	18.86 (25.40)	34.17 (27.72)	< 0.001[1]
Emotional balance	8.76 (12.06)	19.3 (16.30)	< 0.001[1]
Alertness	5.15 (8.69)	9.79 (11.24)	< 0.001[1]
Body care and movement	7.06 (12.56)	16.09 (23.73)	0.002[1]
Ambulation	9.60 (16.53)	22.38 (24.48)	< 0.001[1]
Mobility	4.34 (7.67)	11.19 (13.36)	< 0.001[1]
Communication	2.32 (6.41)	7.91 (12.63)	0.001[1]

SD: Standard Deviation; HCV: Hepatitis C virus; IV: Intravenous; HAD: Hospital Anxiety and Depression Scale; SIP: Sickness Impact Profile. † Other ethnicity includes Hispanic, Native American, Asian and Pacific Islander. [1]Denotes statistically significant *P* value.

as widespread social isolation. This feeling negatively impacted nearly all aspects of their social lives: "Well, it has affected my family, like even my parents and stuff. My mother's afraid to kiss me; she thinks it's like AIDS, you know. It caused a divorce." In 25 additional cases (7% of the total sample), it was the patients themselves who chose to withdraw from families or friends: "When I first found out that I had something wrong with my liver I was very upset and didn't want anyone around me and tried to keep people from me…Right at the moment I have spent a lot of time away from my family. I'm not really wanting to go around them until I find out for sure what's going on."

Underlying reasons for a loss of social support: Three dominant themes emerged when patients explained why their social support had been lessened because of their HCV infection. (1) Transmission of the virus: First, concerns about possible transmission of the virus were described as a major stressor by 84 patients (25% of the total sample). Most of these concerns, voiced by 69 patients (20% of the total sample), focused on sexual partners and family members: "I live with a woman who's deathly afraid of contracting HCV from me, so there's friction there." A smaller number of patients (*n* = 36; 11% of the total sample) were afraid that they might infect friends. Because of fears related to maternal-fetal transmission, 2% of the total sample mentioned concerns about having children, which included the decision to forgo having a family: "(I feel) like I am cootied- like I have cooties. I feel like even if I wanted to have children, I couldn't." However, these often excessive concerns also led to some appropriate precautions. Forty participants (12% of the total sample) stated that they had started using various precautions, including barrier methods for contraception as well as keeping all wounds bandaged, utensils clean, and/or warning others of potential infection risks. (2) Ignorance surrounding the disease: Ignorance, sometimes leading to frank discrimination, was described by 66 participants (19% of the total sample) as directly disrupting relations with family and/or friends. One young man stated: "I lost probably half my friends as they don' t know what the disease is. They just automatically put up a front. I just don't see them anymore-fear of what may happen. So ignorance is how I like to put it." In

conjunction with these feelings, 61 patients (18% of the total sample) had decided that they would not tell others of their disease to decrease the chance of experiencing a negative reaction: "I don't tell anybody 'cause…people are stupid about this disease. Just like they're stupid about AIDS." A small group of patients (n = 11; 3% of the total sample) did not experience discrimination, but rather sensed that those around them simply did not understand that HCV was a serious disease that could affect physical and emotional functioning: "Family members still don' t understand because you don't look sick, so therefore, you should still be able to do everything." (3) Stress caused by the disease: Eighty-three participants (24% of the total sample) experienced problems in their social environment, which they attributed to disease related stress. Such HCV related stress took several forms. One was the unpredictability of the infection's outcome, which left family members and/or friends worrying about the patient: "It's sort of depressing for my family members, because they're all really concerned. I've got daughters and they're worried that I'm going to die." For some patients, a major stressor of living with HCV was their own personal concerns for family and/or friends: "I've got to make sure that my family is provided for, 'cause I don't know if I'm going to croak here anytime."

Another frequently mentioned problem with detrimental effects on relationships was fatigue (n = 49, 14% of the total sample). Treatment or disease related fatigue limited the patient's ability to spend time with others: "Basically I am just non-functioning; cannot maintain relationship with my wife; I cannot work. I have two little boys I am not able to spend quality time with. I can spend time with them, but...I don't have the energy to go out and play baseball with them or any of that stuff."

A final source of stress, especially for families, was the financial burden that came either through a loss of work and/or through the cost of the antiviral medication (n = 16, 5% of the total sample). One patient explained the reaction of her husband to the treatment of her disease: "Not good, it's a lot of strain on him and stress and he's emotionally upset because of everything. And he's had to hold the whole road as far as work and we're in debt $40 000. You know, that's the big one, the debt and money 'cause we don't have insurance. How are we ever going to pay for it?"

DISCUSSION

This is the first analysis to display the frequency and to characterize the nature of the relationship challenges that patients with HCV experience. About one third of this large group of HCV infected patients described a negative impact of their disease on personal relationships. The most devastating consequences were with breakups or major stressors on sexual relations as experienced by about 17% of the participants, and the loss of relationships with family members in an additional 16% of cases. Our results are consistent with prior studies focusing on patients with HIV/AIDS or tuberculosis, who also felt rejected by relatives and friends[24,25]. While all chronic diseases can potentially tax a social support network, a diagnosis with HCV appeared to carry with it unique challenges. Patients feared interactions with others, especially sexual partners, because of the perceived risk that they might infect them. Patients also complained that those close to them lacked information about HCV, that they harbored prejudices or simply did not consider the disease to be "real" and, therefore, serious. As a result, 7% of HCV positive patients in our study personally chose to withdraw from social contact with others.

A similar breakdown occurred outside of the home, with participants reporting that friends had shied away from them. As reported in prior studies, a substantial number of patients experienced frank stigmatization[26-29]. Changes in employment, sometimes attributed to perceived stigmatization[10], sometimes fatigue, created additional strains, as patients were facing financial problems exacerbated by the significant costs of the antiviral treatment.

Concerns about quality of life emerged with patients' estrangement from family and friends. Relationship deterioration can constitute an important stressor, which can limit a patient's ability to cope with a disease[30-32]. Several studies have previously found that a lack of social support and personal coping resources can affect mood and trigger depression[33-36].

Our bivariate analysis is consistent with the above mentioned theoretical considerations. Similar to prior reports, we noted an association between emotional problems, including depression, as measured by the HAD, and lower levels of social support[37-39]. A lower level of support was also associated with higher scores for anxiety, as measured by the HAD, more psychiatric diagnoses as listed in the medical chart, and lower psychological well-being as measured by sub-scales and summary scores of the SIP. While studies have consistently found antiviral therapy to be associated with higher levels of depression[40-45], our cross-sectional data did not show differences in the level of social support of those currently on therapy, as compared to those who were pre or post treatment. This may be surprising at first glance because of the known effects of antiviral therapy on mood. Yet our thematic analysis pointed to specific challenges-transmission risks, unpredictable outcomes, feelings of isolation and the perceived need to withdraw from others-that are not unique to patients undergoing treatment.

Interestingly, patients with lower levels of social support were also more likely to report more severe physical symptoms, as shown in higher SIP scores on Body Care and Movement, Mobility, and Ambulation. While we did not notice a relationship with the presence of cirrhosis or the Child Pugh score, a prognostic indicator for advanced liver disease, most of the patients did not have cirrhosis or had well compensated disease, thus, limiting the discriminating value of these variables. The underlying mechanisms for the relationship between poor social support and physical functioning are unclear[46-49]. One explanation, as noted by patients in our study, was that worse physical functioning limited their ability to meet expectations of family and/or friends.

This study was shaped by the belief that patients' accounts accurately reflected their lived experience of having HCV. As such, the narrative findings were limited to patient recall. However, we maintain that an open account by patients is the best way to understand the breadth and depth of the problems affecting their social support. In addition, we interviewed a very large group of patients with HCV, enabling us to see which themes appeared most dominant, thereby decreasing the anecdotal nature of most qualitative findings.

The study was also cross-sectional in nature, thereby limiting causal evidence of whether social support difficulties actually resulted in worse mental and physical health. Future studies should assess patient's social support level prior to HCV testing in order to determine whether the knowledge of having the disease results in negative interpersonal consequences or whether patients with risk factors for HCV are instead prone to having social difficulties. Finally, the study population was representative of the non-urban Midwest of America. Our age and sex distribution are similar to those reported in many of the prominent studies on HCV[50-53]. Also, our results found no significant association between social support and rural/urban living or ethnicity. However, as the majority of our participants were Caucasians, the study is not an ideal vehicle for examining the role of race or ethnicity in social support.

In conclusion, as far as we are aware, our results are the first to characterize in a large patient sample the negative consequences of carrying a diagnosis of HCV on patients' social support. Providers and case managers need to be aware of the themes expressed in this analysis, as fears of contamination, feelings of isolation or withdrawal from others may reduce the emotional willingness of patients to initiate and remain on antiviral therapy. Structured educational sessions for both the patient and their partners and families could help reduce the misunderstandings expressed here, such as the ignorance of the disease or the view that the disease is somehow not "real," which can further burden the resilience of patients with HCV to cope with their disease.

REFERENCES

1 **Fraenkel L**, McGraw S, Wongcharatrawee S, Garcia-Tsao G. Patients' experiences related to anti-viral treatment for hepatitis C. *Patient Educ Couns* 2006; **62**: 148-155

2 **Buti M**, Wong J, Casado MA, Esteban R. Quality of life and cost-effectiveness of anti-HCV therapy in HIV-infected patients. *J Hepatol* 2006; **44**: S60-S64

3 **Tarantino G**, Conca P, Sorrentino P, Ariello M, Mastrolia M, Di Minno MN. Is the quality of life always depressed in patients with chronic hepatitis C on antiviral therapy? *Eur J Gastroenterol Hepatol* 2005; **17**: 999

4 **Perrillo R**, Rothstein KD, Rubin R, Alam I, Imperial J, Harb G, Hu S, Klaskala W. Comparison of quality of life, work productivity and medical resource utilization of peginterferon alpha 2a vs the combination of interferon alpha 2b plus ribavirin as initial treatment in patients with chronic hepatitis C. *J Viral Hepat* 2004; **11**: 157-165

5 **Schaefer M**, Schmidt F, Folwaczny C, Lorenz R, Martin G, Schindlbeck N, Heldwein W, Soyka M, Grunze H, Koenig A, Loeschke K. Adherence and mental side effects during hepatitis C treatment with interferon alfa and ribavirin in psychiatric risk groups. *Hepatology* 2003; **37**: 443-451

6 **Bernstein D**, Kleinman L, Barker CM, Revicki DA, Green J. Relationship of health-related quality of life to treatment adherence and sustained response in chronic hepatitis C patients. *Hepatology* 2002; **35**: 704-708

7 **Hilsabeck RC**, Hassanein TI, Carlson MD, Ziegler EA, Perry W. Cognitive functioning and psychiatric symptomatology in patients with chronic hepatitis C. *J Int Neuropsychol Soc* 2003; **9**: 847-854

8 **Goulding C**, O'Connell P, Murray FE. Prevalence of fibromyalgia, anxiety and depression in chronic hepatitis C virus infection: relationship to RT-PCR status and mode of acquisition. *Eur J Gastroenterol Hepatol* 2001; **13**: 507-511

9 **Akobeng AK**, Davison S. Quality of life of patients with chronic hepatitis C virus infection. *J Pediatr Gastroenterol Nutr* 2000; **30**: 224-226

10 **Zickmund S**, Ho EY, Masuda M, Ippolito L, LaBrecque DR. "They treated me like a leper". Stigmatization and the quality of life of patients with hepatitis C. *J Gen Intern Med* 2003; **18**: 835-844

11 **Grundy G**, Beeching N. Understanding social stigma in women with hepatitis C. *Nurs Stand* 2004; **19**: 35-39

12 **Schäfer A**, Scheurlen M, Felten M, Kraus MR. Physician-patient relationship and disclosure behaviour in chronic hepatitis C in a group of German outpatients. *Eur J Gastroenterol Hepatol* 2005; **17**: 1387-1394

13 **Morrison A**. A woman with leprosy is in double jeopardy. *Lepr Rev* 2000; **71**: 128-143

14 **Ingram D**, Hutchinson SA. HIV-positive mothers and stigma. *Health Care Women Int* 1999; **20**: 93-103

15 **Rajeswari R**, Muniyandi M, Balasubramanian R, Narayanan PR. Perceptions of tuberculosis patients about their physical, mental and social well-being: a field report from south India. *Soc Sci Med* 2005; **60**: 1845-1853

16 **Cormier M**. The role of hepatitis C support groups. *Gastroenterol Nurs* 2005; **28**: S4-S9

17 **Rowan PJ**, Al-Jurdi R, Tavakoli-Tabasi S, Kunik ME, Satrom SL, El-Serag HB. Physical and psychosocial contributors to quality of life in veterans with hepatitis C not on antiviral therapy. *J Clin Gastroenterol* 2005; **39**: 731-736

18 **Zigmond AS**, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983; **67**: 361-370

19 **Bergner M**, Bobbitt RA, Carter WB, Gilson BS. The Sickness Impact Profile: development and final revision of a health status measure. *Med Care* 1981; **19**: 787-805

20 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383

21 **Zickmund S**, Hillis SL, Barnett MJ, Ippolito L, LaBrecque DR. Hepatitis C virus-infected patients report communication problems with physicians. *Hepatology* 2004; **39**: 999-1007

22 **Miller WL**, Crabtree BF. Clinical research: a multitypology and qualitative road map. In: Crabtree BF, Miller WL, ed. Doing Qualitative Research. London: Sage Press, 1992: 3-32

23 **Landis JR**, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159-174

24 **Kadushin G**. Gay men with AIDS and their families of origin: an analysis of social support. *Health Soc Work* 1996; **21**: 141-149

25 **Kelly P**. Isolation and stigma: the experience of patients with active tuberculosis. *J Community Health Nurs* 1999; **16**: 233-241

26 **Minuk GY**, Gutkin A, Wong SG, Kaita KD. Patient concerns regarding chronic hepatitis C infections. *J Viral Hepat* 2005; **12**: 51-57

27 **Crockett B**, Gifford SM. "Eyes Wide Shut": narratives of women living with hepatitis C in Australia. *Women Health* 2004; **39**: 117-137

28 **Crofts N**, Louie R, Loff B. The next plague: stigmatization and discrimination related to hepatitis C virus infection in Australia. *Health Hum Rights* 1997; **2**: 87-97

29 **Banwell C**, Bammer G, Gifford SM, O'Brien ML. Australian lesbian and bisexual women's health and social experiences of

living with hepatitis C. *Health Care Women Int* 2005; **26**: 340-354
30 **Friedman LC**, Brown AE, Romero C, Dulay MF, Peterson LE, Wehrman P, Whisnand DJ, Laufman L, Lomax J. Depressed mood and social support as predictors of quality of life in women receiving home health care. *Qual Life Res* 2005; **14**: 1925-1929
31 **Bramston P**, Chipuer H, Pretty G. Conceptual principles of quality of life: an empirical exploration. *J Intellect Disabil Res* 2005; **49**: 728-733
32 **Jia H**, Uphold CR, Wu S, Chen GJ, Duncan PW. Predictors of changes in health-related quality of life among men with HIV infection in the HAART era. *AIDS Patient Care STDS* 2005; **19**: 395-405
33 **Wattanakit K**, Williams JE, Schreiner PJ, Hirsch AT, Folsom AR. Association of anger proneness, depression and low social support with peripheral arterial disease: the Atherosclerosis Risk in Communities Study. *Vasc Med* 2005; **10**: 199-206
34 **Jump RL**, Robinson ME, Armstrong AE, Barnes EV, Kilbourn KM, Richards HB. Fatigue in systemic lupus erythematosus: contributions of disease activity, pain, depression, and perceived social support. *J Rheumatol* 2005; **32**: 1699-1705
35 **Kuehner C**, Buerger C. Determinants of subjective quality of life in depressed patients: the role of self-esteem, response styles, and social support. *J Affect Disord* 2005; **86**: 205-213
36 **Chou KL**. Everyday competence and depressive symptoms: social support and sense of control as mediators or moderators? *Aging Ment Health* 2005; **9**: 177-183
37 **Bisschop MI**, Kriegsman DM, Beekman AT, Deeg DJ. Chronic diseases and depression: the modifying role of psychosocial resources. *Soc Sci Med* 2004; **59**: 721-733
38 **Schwartz C**, Frohner R. Contribution of demographic, medical, and social support variables in predicting the mental health dimension of quality of life among people with multiple sclerosis. *Health Soc Work* 2005; **30**: 203-212
39 **McDonnell KA**, Gielen AC, O'Campo P, Burke JG. Abuse, HIV status and health-related quality of life among a sample of HIV positive and HIV negative low income women. *Qual Life Res* 2005; **14**: 945-957
40 **Hauser P**, Khosla J, Aurora H, Laurin J, Kling MA, Hill J, Gulati M, Thornton AJ, Schultz RL, Valentine AD, Meyers CA, Howell CD. A prospective study of the incidence and open-label treatment of interferon-induced major depressive disorder in patients with hepatitis C. *Mol Psychiatry* 2002; **7**: 942-947
41 **Dieperink E**, Ho SB, Tetrick L, Thuras P, Dua K, Willenbring ML. Suicidal ideation during interferon-alpha2b and ribavirin treatment of patients with chronic hepatitis C. *Gen Hosp Psychiatry* 2004; **26**: 237-240
42 **Bonaccorso S**, Puzella A, Marino V, Pasquini M, Biondi M, Artini M, Almerighi C, Levrero M, Egyed B, Bosmans E, Meltzer HY, Maes M. Immunotherapy with interferon-alpha in patients affected by chronic hepatitis C induces an intercorrelated stimulation of the cytokine network and an increase in depressive and anxiety symptoms. *Psychiatry Res* 2001; **105**: 45-55
43 **Bonaccorso S**, Marino V, Puzella A, Pasquini M, Biondi M, Artini M, Almerighi C, Verkerk R, Meltzer H, Maes M. Increased depressive ratings in patients with hepatitis C receiving interferon-alpha-based immunotherapy are related to interferon-alpha-induced changes in the serotonergic system. *J Clin Psychopharmacol* 2002; **22**: 86-90
44 **Kraus MR**, Schäfer A, Faller H, Csef H, Scheurlen M. Psychiatric symptoms in patients with chronic hepatitis C receiving interferon alfa-2b therapy. *J Clin Psychiatry* 2003; **64**: 708-714
45 **Kraus MR**, Schäfer A, Wissmann S, Reimer P, Scheurlen M. Neurocognitive changes in patients with hepatitis C receiving interferon alfa-2b and ribavirin. *Clin Pharmacol Ther* 2005; **77**: 90-100
46 **Shaw BA**, Janevic M. Associations between anticipated support, physical functioning, and education level among a nationally representative sample of older adults. *J Aging Health* 2004; **16**: 539-561
47 **Travis LA**, Lyness JM, Shields CG, King DA, Cox C. Social support, depression, and functional disability in older adult primary-care patients. *Am J Geriatr Psychiatry* 2004; **12**: 265-271
48 **Shen BJ**, McCreary CP, Myers HF. Independent and mediated contributions of personality, coping, social support, and depressive symptoms to physical functioning outcome among patients in cardiac rehabilitation. *J Behav Med* 2004; **27**: 39-62
49 **Patel SS**, Peterson RA, Kimmel PL. The impact of social support on end-stage renal disease. *Semin Dial* 2005; **18**: 98-102
50 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499
51 **Heathcote EJ**, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, Reindollar R, Reddy RK, Wright TL, Lin A, Hoffman J, De Pamphilis J. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N Engl J Med* 2000; **343**: 1673-1680
52 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
53 **Zeuzem S**, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000; **343**: 1666-1672

APPENDIX

1 Can you tell me what your condition is exactly? What physical limitations do you have and when were you initially diagnosed?
2 Could you tell us a little bit about yourself: the things you've done, your roles in life, who you are? What in your life are you most proud of?
3 Can you share with us anything specific that might have caused or helped cause your illness?
4 Have any of your family members had similar health problems? Does this family history worry you?
5 What impact has your physical condition had on the quality of your life? Would you say that your quality of life as compared with 1 year ago is better, about the same, or worse?
6 As you go through this experience, have you begun to think about yourself differently?
7 What has been the hardest thing for you to cope with related to this experience? How do you go about coping with your condition?
8 How does this illness make you feel emotionally? If there was one emotion that you would use to express how you feel, what would that be?
9 Since you were diagnosed, what would you say has been your biggest regret? Would you say you feel emotionally worse since you were diagnosed, about the same as before, or emotionally better off than before?
10 Can you share what health related worries you have? Do you worry more about your health than you do other aspects of your life? Did you share these worries with your doctor or nurse?
11 How would you describe your outlook or thoughts as you look to the future?
12 What are the various things that make your life meaningful for you? What do you turn to when you are in need of strength?

13 **We are interested in the impact this illness has had on your relationships:**
(1) If you were in a crisis, who would you turn to? (2) Do you have a spouse or significant other? How has that person reacted to your situation of being ill? When you think about when you were healthy, would you describe the two of you as closer, somehow further part, or just as close as you always were? (3) Can you describe the level of support you can find in your family? Can you explain why are you able or not able to rely on your family to help you through difficult times? (4) Would you say that you are close to others outside of your family? Why would you say that? What about co-workers, are they understanding and supportive?

14 **Have you encountered any negative judgments from others because of your disease? If yes, can you say who has made you feel this way and under what circumstances?**

15 How has your disease affected your sense of control in your life? What are the situations where you feel you're not in control?

16 What has been your experience with your doctors here or elsewhere? Would you describe it as positive or negative and what has made it positive or negative? What in your eyes makes a good doctor?

17 Do you feel that you can ask your doctor or nurse any question? Do you worry that you may be taking their time with your concerns?

18 Where do you get most of your medical information? Is there a source you tend to rely or believe in the most? In general, are there other things about your health condition that you would like to learn from your doctor?

19 What is the most difficult part of following the treatment the doctor recommended for you? Can you describe situations where you are unable to follow the doctor's treatment, such as taking your medicines, watching your diet, or exercising?

20 Did you feel comfortable with your treatment decisions? Do you feel you had choices in your treatment and that they were respected? Who did you discuss your treatment decisions with; what really helped you to make up your mind?

21 Do you use other or supplemental, herbal, or alternative medicines, and if yes, what are those?

22 If you were to describe yourself as something- it can be anything in this world--an object, an animal, anything-- before when you were healthy and now, what two things would you choose?

23 Is there anything else that you would like to add that would help us to understand your experience?

24 Finally, is there anything you would like us to share with a social worker, a pastor, or your health care team about the way you are feeling?

Interviewing Question Guideline (Bold indicates primary questions used in this analysis)

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Differential effects on apoptosis induction in hepatocyte lines by stable expression of hepatitis B virus X protein

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Abstract

AIM: Hepatitis B virus protein X (HBx) has been shown to be weakly oncogenic *in vitro*. The transforming activities of HBx have been linked with the inhibition of several functions of the tumor suppressor p53. We have studied whether HBx may have different effects on p53 depending on the cell type.

METHODS: We used the human hepatoma cell line HepG2 and the immortalized murine hepatocyte line AML12 and analyzed stably transfected clones which expressed physiological amounts of HBx. P53 was induced by UV irradiation.

RESULTS: The p53 induction by UV irradiation was unaffected by stable expression of HBx. However, the expression of the cyclin kinase inhibitor $p21^{waf/cip/sdi}$ which gets activated by p53 was affected in the HBx transformed cell line AML12-HBx9, but not in HepG2. In AML-HBx9 cells, $p21^{waf/cip/sdi}$-protein expression and $p21^{waf/cip/sdi}$ transcription were deregulated. Furthermore, the process of apoptosis was affected in opposite ways in the two cell lines investigated. While stable expression of HBx enhanced apoptosis induced by UV irradiation in HepG2-cells, apoptosis was decreased in HBx transformed AML12-HBx9. P53 repressed transcription from the HBV enhancer I, when expressed from expression vectors or after induction of endogenous p53 by UV irradiation. Repression by endogenous p53 was partially reversible by stably expressed HBx in both cell lines.

CONCLUSION: Stable expression of HBx leads to deregulation of apoptosis induced by UV irradiation depending on the cell line used. In an immortalized hepatocyte line HBx acted anti-apoptotic whereas expression in a carcinoma derived hepatocyte line HBx enhanced apoptosis.

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Key words: Apoptosis; Hepatitis B virus; Hepatocyte lines

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INTRODUCTION

The epidemiological association between chronic HBV infection and the development of hepatocellular carcinoma is well established[1]. However, the molecular mechanism of transformation by HBV is unsolved. Among many potential factors, a viral regulatory protein named Hepatitis B virus protein X (HBx) is believed to contribute to oncogenesis in conjunction with other mechanisms. Stable expression of HBx showed a weak transforming effect in cell lines of liver origin[2-5], in murine fibroblasts[6,7] and in rat fibroblasts[8-12]. Neither the molecular actions set in motion by HBx during the process of transformation, nor the role of HBx for HBV replication are well understood. HBx is dispensable for replication *in vitro*[13], but under certain conditions HBx can enhance replication *in vitro*[14-16]. In the woodchuck, a related virus of the same family of viruses, the hepadnaviridae[17], the analog of HBx is essential for the establishment of infection[18,19]. A plethora of *in vitro* activities and interactions with cellular partners by HBx have been reported[22,23].

In the development of cancer, the delicate balance between cell proliferation and programmed cell death is often disturbed[24]. Several functions of HBx are linked to transformation: HBx transactivates the transcription of cellular proto-oncogenes[25-27], and is able to override cell

cycle check points[28,29]. As the oncoproteins of many tumor viruses influence the course of apoptosis in order to transform their target cells[30-32], several reports described the influence of stable and transient transfection of HBV products on apoptosis. Transfection of replication competent constructs of HBV[33] and the closely related hepadnavirus woodchuck hepatitis virus[34] enhanced apoptosis induction. This pro-apoptotic activity of hepadnavirus genomes was dependent on the integrity of the HBx-ORF[33,34]. Several groups described a pro-apoptotic activity of HBx after transient and stable transfection[33,34-48] whereas other groups described anti-apoptotic effects of HBx[49-59].

The cellular tumor suppressor protein p53 is one of the key players in the induction of apoptosis after genotoxic events[60,61]. Thus, besides influencing programmed cell death the transforming proteins of DNA tumor viruses target and inactivate p53 in quite diverse and elaborate ways[31,32]. Several lines of evidence connect HBx with a disturbance of p53 functions. HBx binds to p53 *in vitro*[62,63], the intracellular distribution of p53 appears to be affected by HBx[4,64,65], p53-dependent and independent DNA repair functions are affected by HBx[66-70]. Furthermore, HBx relieves the repression of transcription by the tumor suppressor p53 in transient reporter gene assays[71-73], and influences the expression of the p53 induced cyclin kinase inhibitor $p21^{waf/cip/sdi}$[74].

However, most of the experiments in these reports were done in transient transfection or even cell free *in vitro* systems. Because the number of HBx molecules in naturally infected cells is assumed to be low[75-77] and transient transfections of HBx expression constructs yield very high amounts of HBx protein, we established cell lines stably expressing low amounts of HBx. Next, we studied the effect of HBx expression on several functions of p53 induced by stimuli that were designed to yield physiological p53 amounts in our cell lines. Using these *bona fide* natural conditions we were able to find that even low levels of HBx altered or even antagonized p53 functions.

MATERIALS AND METHODS

Cell lines

AML12: The highly differentiated murine hepatocyte line AML12[78] derived from mice transgenic for TGFα can be transformed by HBx[3]. AML12 clones stably transfected with and transformed by HBx and the control cell lines stably transfected with pSV2neo were obtained from N. Fausto. AML12 clones were cultured in Dulbecco's MEM/Nutrient Mix F12 supplemented with 10% FCS, 0.1 μM Dexamethason, and ITS (Insulin, Transferrin, and Selene, 5 μg/mL each).

HepG2: The differentiated but already transformed human hepatoblastoma line HepG2[79] was cultivated in Mixed Medium/10% FCS.

CAT assay

For CAT assays, the cells per well were transfected with Lipofectamine (Life Technologies, Karlsruhe). Detection of CAT protein was done with the CAT-ELISA Kit (Roche, Mannheim) according to the instructions of the manufacturer.

UV irradiation

All cell lines were plated out one day before irradiation. Directly before irradiation, the medium was removed and the cultures were washed with PBS. UV irradiation was done in the UV-Stratalinker (Stratagene, Leimen, Germany) at 254 nm. The cells were exposed to UV after removing the cover of the dishes.

For induction of apoptosis, 1000 cells were plated in triplicates on culture dishes with a diameter of 95 mm. After irradiation with the indicated energy in the stratalinker, the cell cultures were grown for 5-15 d, until colonies visible by eye were detected. After washing with PBS, the colonies were stained and fixed with 1% crystal violet in 20% methanol for 5 min. After washing with H_2O, the surviving colonies were counted. Each experiment was done in triplicate and repeated at least thrice.

Western blot

For the lysis of cells, 450 μL of ice cold lysis buffer were added to each well of a six-well plate for 30 min on ice. The lysis was based on RIPA-buffer (0.01 mol/L Tris-HCl, pH 7.4; 0.15 mol/L NaCl; 1% (w/v) sodium desoxycholate; 0.1% (w/v) SDS; 1% Triton X-100) supplemented with Leupeptin and Aprotinin. To digest disturbing chromosomal DNA 250 U/mL Benzonase (Novagen, Merck, Darmstadt) was added to the lysis buffer. After the incubation period, the lysate was cleared by centrifugation at 20 000 g for 5 min. The protein content of the lysates was determined using the BCA assay and equal amounts of protein (20-50 μg) were separated on an SDS gel. After blotting and incubation with the appropriate primary and secondary antibodies, the membranes were developed using Chemiluminescence Blotting Substrate from Boehringer Mannheim, Germany according to the manufacturer's instructions.

Antibodies

The following antibodies were used for the detection of the indicated proteins: murine p53: AB7 (biotinylated polyclonal sheep antibodies from Oncogene Science, via Dianova, Hamburg, Germany); human p53: mAb PAb1801 (Pharmingen, Hamburg, Germany); murine p21: mAb AB4 (OP76 Oncogene, via Dianova, Hamburg, Germany); human p21: p21 (187) (sc-817), St Cruz Biotechnology.

As secondary antibody, we used POD-labeled anti-murine IgG from donkey (Jackson ImmunoResearch, via Dianova, Hamburg, Germany) or POD-strepatividin (Calbiochem, Bad Soden, Germany) for murine p53.

Northern blot

For Northern blot, total cellular RNA was extracted from the cells using TRIzol (Gibco). Twenty micrograms of DNase I digested RNA was separated on a glyoxal agarose gel (1% in 10 mmol/L Na_2HPO_4) and blotted onto positively charged nylon membrane. The blot was hybridized with a ^{32}P labeled p21-specific probe derived from pCMVp21 (murine).

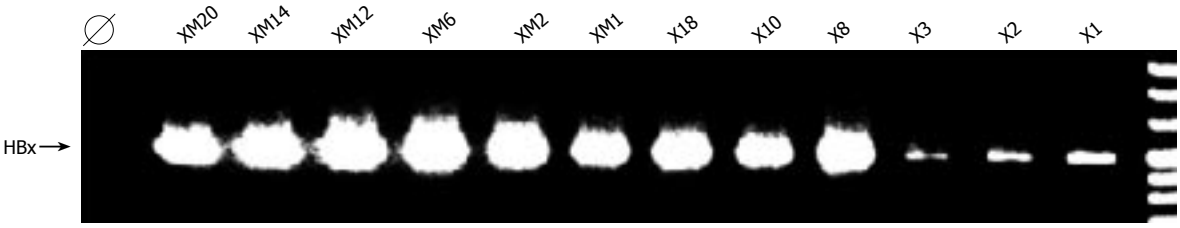

Figure 1 RT-PCR for the detection of HBx-RNA in HepG2 cells stably transfected HBx-expression constructs. Total cellular RNA from clones obtained after stable transfection with expression vector pRCCMVX for wt-HBx (X1, X2, X3, X8, X10, and X18) or for inactive HBx (pRCCMVXM, XM1, XM2, XM6, XM12, XM14, and XM20) was amplified by RT-PCR with primers used for cloning the complete HBx-ORF.

Ribonucleotide protection assay (RPA)

For RPA, an RNA probe complementary to human p21 was transcribed with Maxiscript, Ambion from the construct pCMVsdi[80] according to the instructions of the manufacturer. As an internal control, we used actin transcribed from pTRI-β-Actin (Ambion, Austin, TX, USA).

The RNA probe was purified by electrophoresis in 5% acrylamide/8 mol/L urea gel. The probe (0.5-2 μg) was labeled with biotin using the BrightStar™ Psoralen-Biotin kit (Ambion, Austin, TX, USA).

The RPA was performed with the HybSpeed™ RPA kit from (Ambion, Austin, TX, USA). After digestion of the samples as described in the manual of the kit, the samples were run on 5% PAGE/8 mol/L urea in TBE and blotted onto a positively charged nylon membrane (Amersham, Braunschweig). Signal detection was performed with streptavidin labeled with alkaline phosphatase using the BrightStar™ BioDetect™ kit from Ambion, Austin, TX, USA.

RT-PCR

For RT-PCR, 2 μg of total cellular RNA extracted with Qiagen RNeasy was digested with DNase I for 45 min. One microgram of DNase I digested RNA was reverse transcribed with superscript (Gibco). One quarter of the reverse transcribed RNA was amplified with Taq polymerase (1´ 94℃, 1´ 54℃, 1´ 72℃: × 35 cycles) using HBx-specific primers, which had been used for cloning the complete HBx ORF into pRcCMV.

Constructs

pRcCMVX: The complete HBx ORF of an HBV genotype A genome (serotype adw2; isolate 991; EMBL accession no.: X51970) was cloned with the help of PCR into the *Eco*RI and *Xba*I-site of pCDNA3, Invitrogen, and controlled by sequencing.

pRcCMVXM: Identical to pRcCMVX except for two stop codons introduced by a G to A exchange of nt 1443 (GAA→TAA)[2] and a C to A exchange of nt 1684 (TCA →TAA). Thus, translation of full-length HBx is stopped by exchange of nt 1443 after 23 aa and expression of presumed small HBx proteins is made impossible by exchange of nt 1684, which introduces a stop directly after the third start codon in frame in the HBx-ORF.

PS9: HBV enhancer I (nt 1040-1374 from EMBL accession no.: X51970) was cloned into pBLCAT3[81].

pC53SN3: Expression vector for wt-p53[82]. P53 is expressed from the CMV enhancer in the vector pCMV.

pcDNA3p21 (murine): Murine p21 was cloned by RT-PCR with primers waf-AS and waf-S[33] from total cellular RNA derived from AML12 cells. After *Eco*RI digestion, the amplicon was cloned into pCDNA3. Sequencing gave complete identity with murine p21 from GenBank.

RESULTS

Establishing HBx-expressing HepG2-cell lines

HepG2 cells-a completely transformed hepatoma cell line-were stably transfected with the HBx-expression construct pRcCMVX. As a negative control, HepG2 cells were transfected with the isogenic HBx protein expression-negative construct pRcCMVXM. Clones obtained after G418 treatment were analyzed for the expression of HBx-RNA expression by RT-PCR (Figure 1). 11/13 wt-HBx transfected and 11/11 HBxM transfected clones expressed HBx RNA detectable by RT-PCR. While all HBxM transfected clones expressed similar amounts of HBx RNA as judged by RT-PCR, it appeared as if HBx RNA expression in wt-HBx transfected clones varied significantly (Figure 1). This reminds of the findings in stably HBx transfected clones of rat fibroblast origin, where HBx expression was downregulated in most clones[33].

Clone HepG2X8 expressing wt-HBx and control clone HepG2 XM2 transfected with the HBx-expression minus construct pRcCMVXM expressed similar amounts of HBx-RNA and were chosen to be studied in detail.

In comparison, we analyzed the immortalized murine hepatocyte line AML12[78] and the AML12-clone HBx9 transformed by HBx[3].

Analysis of HBx functions in HBx-expressing AML12 and HepG2 clones

One well-characterized function of HBx is its potential to transactivate virtually any promoter or enhancer. We were unable to detect HBx protein in stably transfected AML12 and HepG2 clones (data not shown and Ref. [3])-a problem encountered by many other groups. In order to show the presence of active HBx, we analyzed whether transcriptional activation by HBx could be detected in cell lines stably expressing HBx RNA. Indeed, the expression from HBV enhancer I was approximately sevenfold stronger in the HBx expressing cell line HepG2-X8 than in the control cell line HepG2-XM2 (Figure 2B). In AML12 cell transactivation by HBx was lower (Figure 2A).

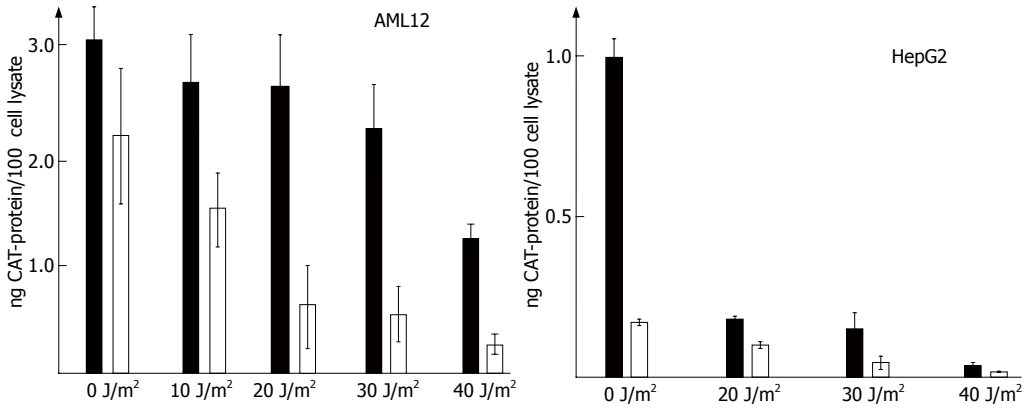

Figure 2 HBx activates the HBV-Enhancer I *in vivo* and can partially counteract a repression by p53 induced by UV irradiation. CAT expression driven by Enhancer I of HBV is indicated as bars. AML12 (A) or HepG2 (B) derived cell lines expressing HBx (HepG2-X8 or AML12-HBx9) are indicated in black and the controls which did not express functional HBx (AML12 or HepG2-XM2) are shown in white bars. The cell lines were transfected with a CAT-expression vector driven by enhancer I of HBV in triplicate. Four hours after transfection, the cultures were irradiated with the indicated dose of UV. The lysis of the cells was done 18 h post irradiation.

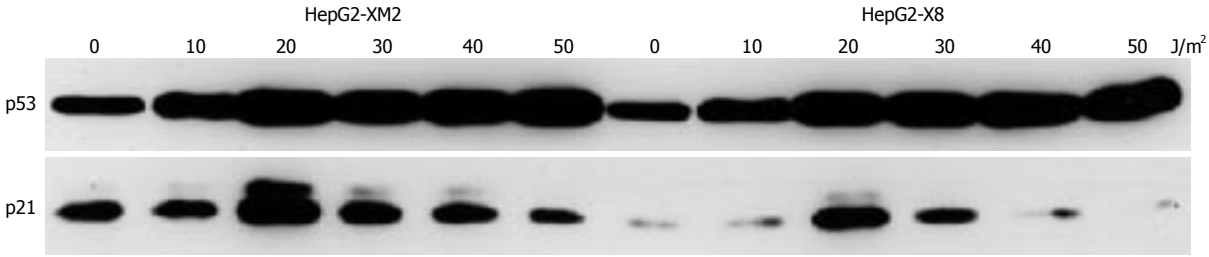

Figure 3 Induction of p53- and p21$^{waf/cip/sdi}$-protein expression in HepG2-derived cell lines after irradiation with UV by immune blot. The dose of UV used for irradiation is indicated. The cells were lysed 18 h after irradiation with UV.

Table 1 Survival of HepG2- and AML12-derived HBx-expressing and control cell lines after irradiation with UV

	0 J/m^2	20 J/m^2	50 J/m^2
HepG2-XM2	100 ± 1.4	17.7 ± 8.0	1.0 ± 0.9
HepG2-X8	100 ± 20.9	1.1 ± 0.6	0.2 ± 0.1
HepG2-XM2	100 ± 11.8	15.8 ± 7.1	1.38 ± 1.5
HepG2-X8	100 ± 20.3	2.7 ± 1.5	0.0
HepG2-XM2	100 ± 15.6	13.5 ± 6.9	3.7 ± 0.8
HepG2-X8	100 ± 7.4	6.8 ± 0.5	1.7 ± 1.2
AML12	100 ± 5.1	4.3 ± 0.6	1.1 ± 1.0
AML12-HBx9	100 ± 14.2	13.5 ± 8.9	6.0 ± 2.2
AML12	100 ± 27.2	17.0 ± 6.3	0.6 ± 0.04
AML12-HBx9	100 ± 3.8	28.0 ± 7.0	6.8 ± 2.4
AML12	100 ± 4.4	11.0 ± 4.1	0.4 ± 0.5
AML12-HBx9	100 ± 4.6	17 ± 5.1	3.4 ± 1.8

The percentage of cells surviving UV-treatment of three independent experiments. For higher reproducibility the HBx-expressing and the control cell lines were irradiated in one batch.

HBx acts pro-apoptotic in HepG2- and anti-apoptotic in AML12-cell lines

To analyze the extent of apoptosis quantitatively, we investigated the survival of the cell lines under investigation after UV irradiation. Five hundred cells in triplicate were plated and irradiated with the dose indicated. Surviving colonies were counted after about 14 d. Table 1 shows that stable expression of HBx acts pro-apoptotic in HepG2-derived and anti-apoptotic in AML12-derived cell lines. The pro-apoptotic effect of HBx in HepG2-X8 was most obvious in cell lines irradiated with 20 J/m^2. For example in one experiment 17.7% ± 8.0 cells of the control cell line HepG2-XM2 survived irradiation with 20 J/m^2, whereas only 1.1% ± 0.6 of identically treated HBx expressing HepG2-X8 cells were able to grow out to visible colonies. AML12 cells were more sensitive against UV irradiation. Expression of HBx acted anti-apoptotic in the HBx expressing AML12 cell line and enhanced the survival rates to similar levels as in HepG2XM control cells.

No influence of HBx on induction of p53 in HepG2- and in AML12-cell lines

p53 is a central protein in the induction of apoptosis[83]. After many sublethal stimuli, a complex set of events takes place that results in an increased expression of p53 in the nucleus. Thus, we analyzed by immune blots of whole cell extracts whether HBx was able to modulate expression of p53 after UV irradiation of the cell lines. Irradiation with increasing doses (0-50 J/m^2) of UV led to enhanced expression of p53 in control and HBx expressing cell lines (Figures 3 and 4). HBx had no effect on the induction of p53 in cell lines of AML12 and HepG2 origin. p53 expression was easily detectable after irradiation with 20 J/m^2 and maximal expression was reached after irradiation with 40-50 J/m^2 in the HepG2 and AML12 cell lines investigated (Figures 3 and 4). These data are in accordance with reports on p53 expression in HepG2-cells[84], human[85], and murine[85,86] fibroblasts and primary rat keratinocytes[87].

We also analyzed the kinetics of p53 induction after UV irradiation with 40 J/m^2. p53 levels increased after 1 h to reach a maximum 18 h after irradiation. Thereafter,

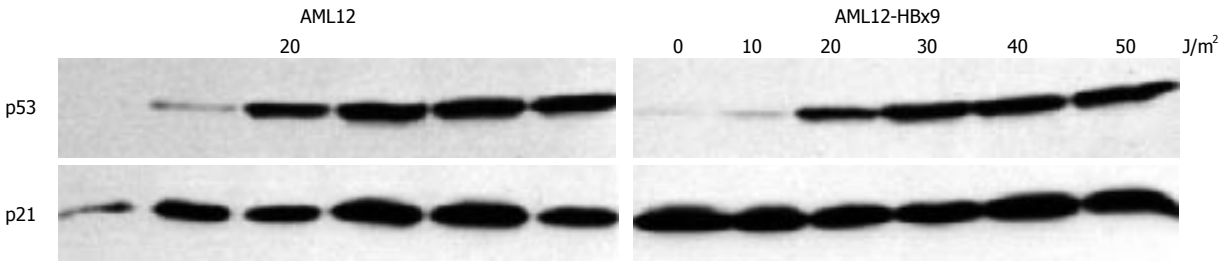

Figure 4 Induction of p53- and p21$^{waf/cip/sdi}$-protein expression in AML12-derived cell lines after irradiation with UV by immune blot. The dose of UV used for irradiation is indicated. The cells were lysed 18 h after irradiation with UV.

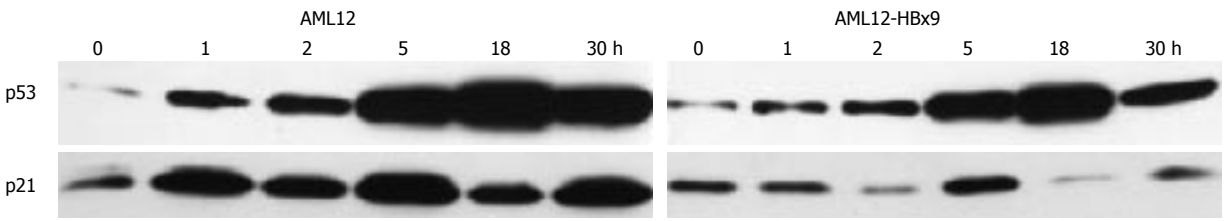

Figure 5 Kinetics of induced p53- and p21$^{waf/cip/sdi}$-protein expression after irradiation with UV by immune blot in AML12 cell lines. The time in hours after irradiation with 40 J/m^2 UV is indicated.

p53 expression decreased (Figure 5). Again, HBx had no influence on the kinetics of p53 induction after UV irradiation.

HBx counteracts a repression by UV irradiation in vivo

We next asked whether HBx may influence functions of p53. One extensively investigated activity of p53 is the ability to repress the transcription of many genes[88]. Thus, we analyzed whether expression of the reporter gene CAT from HBV enhancer I is repressed by p53 - or other factors activated after induction of programmed cell death - *in vivo*. Irradiation of HepG2 and AML12-cell lines with increasing doses of UV caused indeed a strong repression of expression from HBV enhancer I (Figures 2A and B). The repression was highest at 40 J/m^2 (Figures 2A and B), the UV dose leading to maximum expression of p53 (Figures 2 and 3). However, a strong reduction of CAT-repression could already be observed at the lowest UV dose of 10 J/m^2 administered. Interestingly, in the tumor cell line HepG2 (Figure 2B), UV irradiation caused a much stronger repression of expression from enhancer I than in AML12 cells (Figure 2A).

In HBx expressing cell lines, the UV-induced repression was partially counteracted. This anti-repressive activity of HBx was strongest in AML12-HBx9 and still significant in HepG2 cells (Figures 2A and B). In the AML-cell line HBx9 irradiated with 40 J/m^2 the CAT-expression was 4.9-fold higher than in the parental cell line AML12 without HBx. Thus, HBx seems to be able to alleviate transcriptional repression by p53.

Induction of p53 and p21$^{waf/cip/sdi}$ by UV irradiation

Because we observed that HBx influenced the extent of transcriptional repression by p53 we also wanted to analyze if HBx had any influence on transcriptional activation by p53. After UV irradiation, p53 is stabilized[89] and acts as a transcription factor by binding to the promoter of certain target genes leading to enhanced transcription from these promoters, for example of the cyclin kinase inhibitor p21$^{waf/cip/sdi}$[90]. Thus, after UV irradiation, the expression of p21$^{waf/cip/sdi}$ is enhanced[86,91]. We therefore analyzed if stable expression of HBx influenced p53's transcriptional activation of the endogenous p21$^{waf/cip/sdi}$-promoter. UV irradiation of the cell lines with increasing doses of UV led to an increase of p21$^{waf/cip/sdi}$ protein expression (Figures 3 and 4). As reported by other groups, irradiation with high doses of 40-50 J/m^2 UV again caused a decline of the induced p21$^{waf/cip/sdiwaf}$ protein expression[86,91]. An analysis of the temporal regulation of p21$^{waf/cip/sdiwaf}$ protein expression (Figure 5) after UV irradiation in AML12 cells showed an undulation of p21$^{waf/cip/sdiwaf}$ protein expression in the parental cell line and the HBx transformed clone 9. A similar phenomenon was described in the mouse liver after 70% partial hepatectomy[92]. However, these authors analyzed RNA expression and different time points. Interestingly, stable expression of HBx in HepG2 led to a lower expression of p21$^{waf/cip/sdi}$-protein. However, in the HBx transformed cell line AML12-HBx9, the level of p21$^{waf/cip/sdi}$-protein expression after UV irradiation changed from experiment to experiment and no reproducible data for p21 could be obtained, although p53-expression from identical blots (upper lane) gave reproducible results (Figures 4 and 5). It appeared as if the expression of p21$^{waf/cip/sdi}$-protein was deregulated in a seemingly random way by HBx because p21$^{waf/cip/sdi}$-protein expression was reproducible in the parental cell line (Figures 4 and 5).

Butz *et al* had described an uncoupling of the regularly inducible p21$^{waf/cip/sdi}$-RNA expression from the deregulated p21$^{waf/cip/sdi}$-protein expression upon genotoxic stress in some cell lines[109]. Thus, we analyzed the induction of p21$^{waf/cip/sdi}$-RNA in the HBx transfected cell lines. Indeed, analysis of p21$^{waf/cip/sdi}$-RNA expression in the hepatocyte

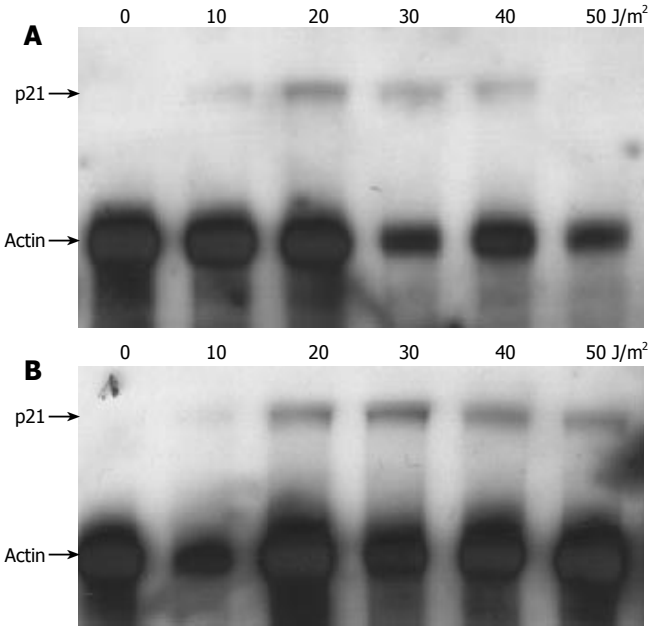

Figure 6 Detection of p21$^{waf/cip/sdi}$-RNA by ribonuclease protection assay in the cell line HepG2-XM2 (**A**) and -HBx8 (**B**). Total cellular RNA was isolated from cell cultures irradiated with the indicated dose of UV 18 h after irradiation. As internal control actin was detected by RPA.

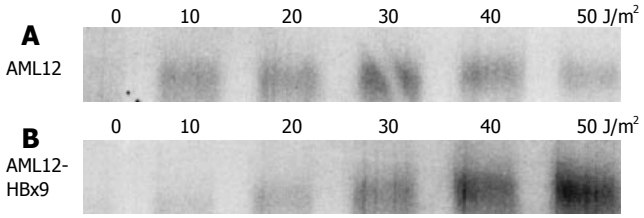

Figure 7 Detection of p21$^{waf/cip/sdi}$-RNA by northern blot in the cell line AML12 (**A**) and the HBx-transformed clone AML12-HBx9 (**B**). Total cellular RNA was isolated from cell cultures irradiated with the indicated dose of UV 18 h after irradiation.

lines under investigation gave reproducible results. Irradiation with UV increased the expression of p21$^{waf/cip/sdi}$-RNA in the HBx-expressing cell lines HepG2-X8 and the control cell lines HepG2-XM2 and AML12 to reach a peak at 20-30 J/m^2 to decrease thereafter (Figures 6 and 7). Analysis of p21$^{waf/cip/sdi}$-RNA expression in the HBx expressing clone AML12-HBx9 - in contrast to analysis of p21$^{waf/cip/sdi}$-protein expression - gave reproducible results. However, no decline of p21$^{waf/cip/sdi}$-RNA expression was observed at higher doses of UV irradiation as with the other three cell lines under investigation (Figure 7). Quite in contrast, in the HBx-expressing clone AML12-HBx9 the amount of p21$^{waf/cip/sdi}$-RNA increased continuously even at the highest dose of UV irradiation of 50 UV J/m^2. Thus, HBx strongly influenced the regulation of p21$^{waf/cip/sdi}$ expression in the HBx-transformed cell line AML12-HBx9.

CONCLUSION

One of the most important functions of p53 is the induction of programmed cell death in order to maintain the integrity of the genome by inhibiting the survival of cells with damaged DNA[93]. To analyze the integrity of p53 function in cell lines stably expressing HBx RNA, we investigated several p53 activities during induction of apoptosis by UV. Irradiation with 20 J/m^2 quite uniformly induced massive cell death in all cell lines analyzed. The cell death was due to apoptosis because we observed nucleosomal DNA fragmentation in all cell lines under investigation (data not shown).

However, we observed that induction of apoptosis was enhanced in HBx expressing cell lines of HepG2-origin and reduced in HBx expressing cell lines of AML12-origin. These opposing effects of HBx are in agreement with the published results on the influence of HBx on programmed cell death. Several groups described a pro-apoptotic activity of HBx after transient and stable transfection[33,34-48] whereas other groups described anti-apoptotic effects of HBx[49-59].

We thus set out to study key events in the induction of apoptosis after UV irradiation. We investigated whether HBx had an effect on the induction of p53 in our cell lines. No evidence was found that HBx influenced the induction of p53 by UV. Neither p53's expression by stabilization of p53 as reported for adenovirus E1A or the large T-Antigen of SV40[94] was increased, nor was p53's expression decreased in the presence of HBx as described for E6 of the papilloma viruses[95]. Our results agree with reports from Refs. [85] and [97] who also did not find altered p53 induction or expression in HBV expressing HepG2 cells.

A widely accepted hypothesis assumes that a higher expression of the cyclin kinase inhibitor p21$^{waf/cip/sdi}$ after DNA damage gives an advantage for survival because cells arrest in G_1 and gain time to repair DNA damage instead of succumbing to apoptosis[88]. DNA damage induced in cell lines with a targeted deletion of the p21$^{waf/cip/sdi}$-gene led to programmed cell death, whereas the wt-cell line survived in cell cycle arrest[97]. After induction, p53 binds as a transcription factor to the promoter of p21$^{waf/cip/sdi}$ and increases its transcription[90]. Our results on induction of p21$^{waf/cip/sdi}$-protein and RNA by UV irradiation in the control cell lines (AML12 and HepG2-XM2) are in accordance with data from literature[86,87,91]. Interestingly, the HBx-expressing clone HepG2-clone X8 expressed less p21$^{waf/cip/sdi}$ than the control. A decrease as well as an increase of p21$^{waf/cip/sdi}$ expression in response to HBV infection has been described in literature. In microarrays, an enhanced expression of p21$^{waf/cip/sdi}$ was seen in Hep3B and HepG2 lines stably expressing HBx[96,98] and in the HBV expressing cell line HepG2.2.15[99], decreased expression of p21$^{waf/cip/sdi}$ was seen in HBV associated HCC[100], in transiently transfected primary human hepatocytes[101] and in two liver samples from patients with chronic HBV infection[101].

The reason for the differential effect of HBx on the expression of p21$^{waf/cip/sdi}$ in AML12 and HepG2 cell lines is unknown. However, transduction of HBx into primary human hepatocytes and stably transformed hepatocyte lines had reciprocal effects on the expression of several target genes[101]. It appears possible that stable expression of HBx in the immortalized AML12 cell line mimics the situation found in acutely infected liver

with deregulated and in some circumstances enhanced $p21^{waf/cip/sdi}$ expression[100], whereas stable expression of HBx in hepatoma cell line HepG2 with slightly decreased expression of $p21^{waf/cip/sdi}$ mirrors the situation found in HBV associated HCC[100], i.e. in the endpoint of hepatocarcinogenesis.

It appears possible that HBx transactivates the enhancer of $p21^{waf/cip/sdi}$ in AML12 cells directly, as has been reported for other cell lines[74,96]. In addition, HBx may enhance the physiological activation of $p21^{waf/cip/sdi}$ by p53. Haviv *et al*[102] have shown that HBx can drastically enhance p53 mediated transcriptional activation. In consequence, HBx expressing AML12 clones would express more $p21^{waf/cip/sdi}$ than controls. Furthermore, HBx may have a stronger effect on the activation of the transcription factor *ets* in AML12 cells than in HepG2 cells, as *ets* has been suggested to play a crucial role in the transactivation of the $p21^{waf/cip/sdi}$ enhancer by HBx[96]. These assumptions would be in agreement with the higher and deregulated expression of $p21^{waf/cip/sdi}$-RNA in the HBx expressing AML12 cell line. An enhanced expression of $p21^{waf/cip/sdi}$ in the HBx transformed cell line AML12-HBx9 reminds of findings in HTLV-1 transformed cell lines[103,104]. Tax, the transforming protein of HTLV-1 virus[105] has many functional similarities with HBx[106]. The oncoprotein E7 of the papilloma viruses also enhances the expression of $p21^{waf/cip/sdi}$[107,108]. As described for otherwise unrelated cell lines[109], we also observed that the expression of $p21^{waf/cip/sdi}$-protein was deregulated and did not seem to correlate with $p21^{waf/cip/sdi}$-RNA-expression in HBx-expressing cell line AML12-HBx9 (Figures 4, 5 and 7).

Our results indicate that HBx is able to partially relieve repression of expression from the HBV enhancer I by physiological amounts of p53 as observed after induction by UV irradiation (Figure 2). Other groups reported complete relief of transcriptional repression by p53 after transient transfection of HBx[71] or HBV-dimers[72] or in *in vitro* transcription assays using *in vitro* translated p53 and HBx proteins[73]. However, transient transfection of HBx expression constructs leads to high expression of HBx, whereas stable expression of HBx as in our experiments only leads to low level expression of HBx that may be more comparable to the natural situation in tissue of chronically infected individuals where detection of HBx is rather difficult because of its low level of expression[75-77]. Moreover, in transient assays using expression vectors for p53 and HBx, a complete relief of transcriptional repression by p53 was only seen when 100-fold more HBx expression vector than p53 was used[71]. When equal amounts of expression constructs were used, HBx was not able to relieve transcriptional repression by p53[71,110]. Our results indicate that stable expression of HBx in hepatocyte lines antagonizes functions of physiological doses of p53 induced by UV and confirm the results of transient transfections[71,72].

In summary, we found several p53 linked activities that were altered in cell lines stably expressing HBx. By use of conditions which closely match the natural situation, we were able to show that HBx is able to alleviate some of the p53 induced effects taking place after the induction of apoptosis by UV irradiation. The differences we observe between the HBx expressing immortalized hepatocyte line AML12 and the hepatoma cell line HepG2 are due to a differential activation of intracellular signal transduction pathways - as found in inducible HBx expressing AML12-clones[5,111] - and will be the subject of further investigations.

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REFERENCES

1 **Bréchot C**, Jaffredo F, Lagorce D, Gerken G, Meyer zum Büschenfelde K, Papakonstontinou A, Hadziyannis S, Romeo R, Colombo M, Rodes J, Bruix J, Williams R, Naoumov N. Impact of HBV, HCV and GBV-C/HGV on hepatocellular carcinomas in Europe: results of a European concerted action. *J Hepatol* 1998; **29**: 173-183

2 **Seifer M**, Höhne M, Schaefer S, Gerlich WH. In vitro tumorigenicity of hepatitis B virus DNA and HBx protein. *J Hepatol* 1991; **13** Suppl 4: S61-S65

3 **Oguey D**, Dumenco LL, Pierce RH, Fausto N. Analysis of the tumorigenicity of the X gene of hepatitis B virus in a nontransformed hepatocyte cell line and the effects of cotransfection with a murine p53 mutant equivalent to human codon 249. *Hepatology* 1996; **24**: 1024-1033

4 **Schaefer S**, Seifer M, Grimmsmann T, Fink L, Wenderhold S, Höhne MW, Gerlich WH. Properties of tumour suppressor p53 in murine hepatocyte lines transformed by hepatitis B virus X protein. *J Gen Virol* 1998; **79** (Pt 4): 767-777

5 **Tarn C**, Bilodeau ML, Hullinger RL, Andrisani OM. Differential immediate early gene expression in conditional hepatitis B virus pX-transforming versus nontransforming hepatocyte cell lines. *J Biol Chem* 1999; **274**: 2327-2336

6 **Shirakata Y**, Kawada M, Fujiki Y, Sano H, Oda M, Yaginuma K, Kobayashi M, Koike K. The X gene of hepatitis B virus induced growth stimulation and tumorigenic transformation of mouse NIH3T3 cells. *Jpn J Cancer Res* 1989; **80**: 617-621

7 **Seifer M**, Höhne M, Schaefer S, Gerlich WH. Malignant transformation of immortalized cells by hepatitis B virus DNA. In: Hollinger F, Lemon S, Margolis H, editors. Viral Hepatitis-1990. New York: Williams and Wilkins, 1991: 586-588

8 **Gottlob K**, Pagano S, Levrero M, Graessmann A. Hepatitis B virus X protein transcription activation domains are neither required nor sufficient for cell transformation. *Cancer Res* 1998; **58**: 3566-3570

9 **Buendia MA**, Paterlini P, Tiollais P, Bréchot C. Hepatocellular carcinoma: molecular aspects. In: Zuckerman AJ, Thomas CT, editors. Viral hepatitis. 2nd ed. London: Churchill Livingstone, 1998: 179-200

10 **Bréchot C**, Gozuacik D, Murakami Y, Paterlini-Bréchot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Semin Cancer Biol* 2000; **10**: 211-231

11 **Schaefer S**. Hepatitis B Virus in Experimental Carcinogenesis. In: Grand RJA, editor. Viruses, cell transformation and cancer. Greenwich: Elsevier, 2001: 193-228

12 **Block TM**, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene* 2003; **22**: 5093-5107

13 **Blum HE**, Zhang ZS, Galun E, von Weizsäcker F, Garner B, Liang TJ, Wands JR. Hepatitis B virus X protein is not central

to the viral life cycle in vitro. *J Virol* 1992; **66**: 1223-1227
14 **Bouchard MJ**, Puro RJ, Wang L, Schneider RJ. Activation and inhibition of cellular calcium and tyrosine kinase signaling pathways identify targets of the HBx protein involved in hepatitis B virus replication. *J Virol* 2003; **77**: 7713-7719
15 **Hafner A**, Brandenburg B, Hildt E. Reconstitution of gene expression from a regulatory-protein-deficient hepatitis B virus genome by cell-permeable HBx protein. *EMBO Rep* 2003; **4**: 767-773
16 **Zhang Z**, Protzer U, Hu Z, Jacob J, Liang TJ. Inhibition of cellular proteasome activities enhances hepadnavirus replication in an HBX-dependent manner. *J Virol* 2004; **78**: 4566-4572
17 **Burrell CJ**, Chisari FV, Gerlich WH, Gowans EJ, Howard CR, Kann M. Hepadnaviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, editors. Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press, 2000: 325- 334
18 **Chen HS**, Kaneko S, Girones R, Anderson RW, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, Purcell RH, Miller RH. The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. *J Virol* 1993; **67**: 1218-1226
19 **Zoulim F**, Saputelli J, Seeger C. Woodchuck hepatitis virus X protein is required for viral infection in vivo. *J Virol* 1994; **68**: 2026-2030
20 **Yen TS**. Hepadnaviral X Protein:Review of Recent Progress. *J Biomed Sci* 1996; **3**: 20-30
21 **Madden CR**, Slagle BL. Stimulation of cellular proliferation by hepatitis B virus X protein. *Dis Markers* 2001; **17**: 153-157
22 **Murakami S**. Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 2001; **36**: 651-660
23 **Waris G**, Siddiqui A. Regulatory mechanisms of viral hepatitis B and C. *J Biosci* 2003; **28**: 311-321
24 **Kanzler S**, Galle PR. Apoptosis and the liver. *Semin Cancer Biol* 2000; **10**: 173-184
25 **Avantaggiati ML**, Natoli G, Balsano C, Chirillo P, Artini M, De Marzio E, Collepardo D, Levrero M. The hepatitis B virus (HBV) pX transactivates the c-fos promoter through multiple cis-acting elements. *Oncogene* 1993; **8**: 1567-1574
26 **Kekulé AS**, Lauer U, Weiss L, Luber B, Hofschneider PH. Hepatitis B virus transactivator HBx uses a tumour promoter signalling pathway. *Nature* 1993; **361**: 742-745
27 **Twu JS**, Lai MY, Chen DS, Robinson WS. Activation of protooncogene c-jun by the X protein of hepatitis B virus. *Virology* 1993; **192**: 346-350
28 **Koike K**, Moriya K, Yotsuyanagi H, Iino S, Kurokawa K. Induction of cell cycle progression by hepatitis B virus HBx gene expression in quiescent mouse fibroblasts. *J Clin Invest* 1994; **94**: 44-49
29 **Benn J**, Schneider RJ. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. *Proc Natl Acad Sci USA* 1995; **92**: 11215-11219
30 **Roulston A**, Marcellus RC, Branton PE. Viruses and apoptosis. *Annu Rev Microbiol* 1999; **53**: 577-628
31 **Butel JS**. Viral carcinogenesis: revelation of molecular mechanisms and etiology of human disease. *Carcinogenesis* 2000; **21**: 405-426
32 **Lowe SW**, Lin AW. Apoptosis in cancer. *Carcinogenesis* 2000; **21**: 485-495
33 **Schuster R**, Gerlich WH, Schaefer S. Induction of apoptosis by the transactivating domains of the hepatitis B virus X gene leads to suppression of oncogenic transformation of primary rat embryo fibroblasts. *Oncogene* 2000; **19**: 1173-1180
34 **Su F**, Schneider RJ. Hepatitis B virus HBx protein sensitizes cells to apoptotic killing by tumor necrosis factor alpha. *Proc Natl Acad Sci USA* 1997; **94**: 8744-8749
35 **Chirillo P**, Pagano S, Natoli G, Puri PL, Burgio VL, Balsano C, Levrero M. The hepatitis B virus X gene induces p53-mediated programmed cell death. *Proc Natl Acad Sci USA* 1997; **94**: 8162-8167
36 **Kim H**, Lee H, Yun Y. X-gene product of hepatitis B virus induces apoptosis in liver cells. *J Biol Chem* 1998; **273**: 381-385
37 **Terradillos O**, Pollicino T, Lecoeur H, Tripodi M, Gougeon ML, Tiollais P, Buendia MA. p53-independent apoptotic effects of the hepatitis B virus HBx protein in vivo and in vitro. *Oncogene* 1998; **17**: 2115-2123
38 **Bergametti F**, Prigent S, Luber B, Benoit A, Tiollais P, Sarasin A, Transy C. The proapoptotic effect of hepatitis B virus HBx protein correlates with its transactivation activity in stably transfected cell lines. *Oncogene* 1999; **18**: 2860-2871
39 **Shintani Y**, Yotsuyanagi H, Moriya K, Fujie H, Tsutsumi T, Kanegae Y, Kimura S, Saito I, Koike K. Induction of apoptosis after switch-on of the hepatitis B virus X gene mediated by the Cre/loxP recombination system. *J Gen Virol* 1999; **80** (Pt 12): 3257-3265
40 **Chang SF**, Netter HJ, Hildt E, Schuster R, Schaefer S, Hsu YC, Rang A, Will H. Duck hepatitis B virus expresses a regulatory HBx-like protein from a hidden open reading frame. *J Virol* 2001; **75**: 161-170
41 **Schuster R**, Hildt E, Chang SF, Terradillos O, Pollicino T, Lanford R, Gerlich WH, Will H, Schaefer S. Conserved transactivating and pro-apoptotic functions of hepadnaviral X protein in ortho- and avihepadnaviruses. *Oncogene* 2002; **21**: 6606-6613
42 **Song CZ**, Bai ZL, Song CC, Wang QW. Aggregate formation of hepatitis B virus X protein affects cell cycle and apoptosis. *World J Gastroenterol* 2003; **9**: 1521-1524
43 **Su F**, Theodosis CN, Schneider RJ. Role of NF-kappaB and myc proteins in apoptosis induced by hepatitis B virus HBx protein. *J Virol* 2001; **75**: 215-225
44 **Chami M**, Ferrari D, Nicotera P, Paterlini-Bréchot P, Rizzuto R. Caspase-dependent alterations of Ca2+ signaling in the induction of apoptosis by hepatitis B virus X protein. *J Biol Chem* 2003; **278**: 31745-31755
45 **Kim KH**, Seong BL. Pro-apoptotic function of HBV X protein is mediated by interaction with c-FLIP and enhancement of death-inducing signal. *EMBO J* 2003; **22**: 2104-2116
46 **Shirakata Y**, Koike K. Hepatitis B virus X protein induces cell death by causing loss of mitochondrial membrane potential. *J Biol Chem* 2003; **278**: 22071-22078
47 **Chen HY**, Tang NH, Li XJ, Zhang SJ, Chen ZX, Wang XZ. Transfection and expression of hepatitis B virus x gene and its effect on apoptosis in HL-7702 cells. *World J Gastroenterol* 2004; **10**: 959-964
48 **Tanaka Y**, Kanai F, Kawakami T, Tateishi K, Ijichi H, Kawabe T, Arakawa Y, Kawakami T, Nishimura T, Shirakata Y, Koike K, Omata M. Interaction of the hepatitis B virus X protein (HBx) with heat shock protein 60 enhances HBx-mediated apoptosis. *Biochem Biophys Res Commun* 2004; **318**: 461-469
49 **Wang XW**, Gibson MK, Vermeulen W, Yeh H, Forrester K, Stürzbecher HW, Hoeijmakers JH, Harris CC. Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. *Cancer Res* 1995; **55**: 6012-6016
50 **Elmore LW**, Hancock AR, Chang SF, Wang XW, Chang S, Callahan CP, Geller DA, Will H, Harris CC. Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis. *Proc Natl Acad Sci USA* 1997; **94**: 14707-14712
51 **Gottlob K**, Fulco M, Levrero M, Graessmann A. The hepatitis B virus HBx protein inhibits caspase 3 activity. *J Biol Chem* 1998; **273**: 33347-33353
52 **Diao J**, Khine AA, Sarangi F, Hsu E, Iorio C, Tibbles LA, Woodgett JR, Penninger J, Richardson CD. X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. *J Biol Chem* 2001; **276**: 8328-8340
53 **Shih WL**, Kuo ML, Chuang SE, Cheng AL, Doong SL. Hepatitis B virus X protein inhibits transforming growth factor-beta -induced apoptosis through the activation of phosphatidylinositol 3-kinase pathway. *J Biol Chem* 2000; **275**: 25858-25864
54 **Huo TI**, Wang XW, Forgues M, Wu CG, Spillare EA, Giannini C, Brechot C, Harris CC. Hepatitis B virus X mutants derived from human hepatocellular carcinoma retain the ability to abrogate p53-induced apoptosis. *Oncogene* 2001; **20**: 3620-3628

55 **Kim YC**, Song KS, Yoon G, Nam MJ, Ryu WS. Activated ras oncogene collaborates with HBx gene of hepatitis B virus to transform cells by suppressing HBx-mediated apoptosis. *Oncogene* 2001; **20**: 16-23

56 **Lee YI**, Kang-Park S, Do SI, Lee YI. The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J Biol Chem* 2001; **276**: 16969-16977

57 **Li D**, Chen X, Zhang W. The inhibition of apoptosis of hepatoma cells induced by HBx is mediated by up-regulation of survivin expression. *J Huazhong Univ Sci Technolog Med Sci* 2003; **23**: 383-386

58 **Shih WL**, Kuo ML, Chuang SE, Cheng AL, Doong SL. Hepatitis B virus X protein activates a survival signaling by linking SRC to phosphatidylinositol 3-kinase. *J Biol Chem* 2003; **278**: 31807-31813

59 **Kalra N**, Kumar V. c-Fos is a mediator of the c-myc-induced apoptotic signaling in serum-deprived hepatoma cells via the p38 mitogen-activated protein kinase pathway. *J Biol Chem* 2004; **279**: 25313-25319

60 **Rich T**, Allen RL, Wyllie AH. Defying death after DNA damage. *Nature* 2000; **407**: 777-783

61 **Slee EA**, O'Connor DJ, Lu X. To die or not to die: how does p53 decide? *Oncogene* 2004; **23**: 2809-2818

62 **Wang XW**, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proc Natl Acad Sci USA* 1994; **91**: 2230-2234

63 **Truant R**, Antunovic J, Greenblatt J, Prives C, Cromlish JA. Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. *J Virol* 1995; **69**: 1851-1859

64 **Ueda H**, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Feitelson MA, Jay G. Functional inactivation but not structural mutation of p53 causes liver cancer. *Nat Genet* 1995; **9**: 41-47

65 **Takada S**, Kaneniwa N, Tsuchida N, Koike K. Cytoplasmic retention of the p53 tumor suppressor gene product is observed in the hepatitis B virus X gene-transfected cells. *Oncogene* 1997; **15**: 1895-1901

66 **Prost S**, Ford JM, Taylor C, Doig J, Harrison DJ. Hepatitis B x protein inhibits p53-dependent DNA repair in primary mouse hepatocytes. *J Biol Chem* 1998; **273**: 33327-33332

67 **Groisman IJ**, Koshy R, Henkler F, Groopman JD, Alaoui-Jamali MA. Downregulation of DNA excision repair by the hepatitis B virus-x protein occurs in p53-proficient and p53-deficient cells. *Carcinogenesis* 1999; **20**: 479-483

68 **Jia L**, Wang XW, Harris CC. Hepatitis B virus X protein inhibits nucleotide excision repair. *Int J Cancer* 1999; **80**: 875-879

69 **Sohn S**, Jaitovitch-Groisman I, Benlimame N, Galipeau J, Batist G, Alaoui-Jamali MA. Retroviral expression of the hepatitis B virus x gene promotes liver cell susceptibility to carcinogen-induced site specific mutagenesis. *Mutat Res* 2000; **460**: 17-28

70 **Jaitovich-Groisman I**, Benlimame N, Slagle BL, Perez MH, Alpert L, Song DJ, Fotouhi-Ardakani N, Galipeau J, Alaoui-Jamali MA. Transcriptional regulation of the TFIIH transcription repair components XPB and XPD by the hepatitis B virus x protein in liver cells and transgenic liver tissue. *J Biol Chem* 2001; **276**: 14124-14132

71 **Takada S**, Kaneniwa N, Tsuchida N, Koike K. Hepatitis B virus X gene expression is activated by X protein but repressed by p53 tumor suppressor gene product in the transient expression system. *Virology* 1996; **216**: 80-89

72 **Doitsh G**, Shaul Y. HBV transcription repression in response to genotoxic stress is p53-dependent and abrogated by pX. *Oncogene* 1999; **18**: 7506-7513

73 **Ogden SK**, Lee KC, Barton MC. Hepatitis B viral transactivator HBx alleviates p53-mediated repression of alpha-fetoprotein gene expression. *J Biol Chem* 2000; **275**: 27806-27814

74 **Uchida T**, Takahashi K, Tatsuno K, Dhingra U, Eliason JF. Inhibition of hepatitis-B-virus core promoter by p53: implications for carcinogenesis in hepatocytes. *Int J Cancer* 1996; **67**: 892-897

75 **Dandri M**, Schirmacher P, Rogler CE. Woodchuck hepatitis virus X protein is present in chronically infected woodchuck liver and woodchuck hepatocellular carcinomas which are permissive for viral replication. *J Virol* 1996; **70**: 5246-5254

76 **Dandri M**, Petersen J, Stockert RJ, Harris TM, Rogler CE. Metabolic labeling of woodchuck hepatitis B virus X protein in naturally infected hepatocytes reveals a bimodal half-life and association with the nuclear framework. *J Virol* 1998; **72**: 9359-9364

77 **Su Q**, Schröder CH, Hofmann WJ, Otto G, Pichlmayr R, Bannasch P. Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. *Hepatology* 1998; **27**: 1109-1120

78 **Wu JC**, Merlino G, Fausto N. Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. *Proc Natl Acad Sci USA* 1994; **91**: 674-678

79 **Aden DP**, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 1979; **282**: 615-616

80 **Noda A**, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 1994; **211**: 90-98

81 **Luckow B**, Schütz G. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res* 1987; **15**: 5490

82 **Kern SE**, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* 1992; **256**: 827-830

83 **Hall PA**, Lane DP. Tumor suppressors: a developing role for p53? *Curr Biol* 1997; **7**: R144-147

84 **Puisieux A**, Ji J, Guillot C, Legros Y, Soussi T, Isselbacher K, Ozturk M. p53-mediated cellular response to DNA damage in cells with replicative hepatitis B virus. *Proc Natl Acad Sci USA* 1995; **92**: 1342-1346

85 **Lu X**, Lane DP. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell* 1993; **75**: 765-778

86 **Poon RY**, Jiang W, Toyoshima H, Hunter T. Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. *J Biol Chem* 1996; **271**: 13283-13291

87 **Petrocelli T**, Poon R, Drucker DJ, Slingerland JM, Rosen CF. UVB radiation induces p21Cip1/WAF1 and mediates G1 and S phase checkpoints. *Oncogene* 1996; **12**: 1387-1396

88 **May P**, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* 1999; **18**: 7621-7636

89 **Lakin ND**, Jackson SP. Regulation of p53 in response to DNA damage. *Oncogene* 1999; **18**: 7644-7655

90 **el-Deiry WS**, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993; **75**: 817-825

91 **Lu X**, Burbidge SA, Griffin S, Smith HM. Discordance between accumulated p53 protein level and its transcriptional activity in response to u.v. radiation. *Oncogene* 1996; **13**: 413-418

92 **Albrecht JH**, Meyer AH, Hu MY. Regulation of cyclin-dependent kinase inhibitor p21(WAF1/Cip1/Sdi1) gene expression in hepatic regeneration. *Hepatology* 1997; **25**: 557-563

93 **Lane DP**. Cancer. p53, guardian of the genome. *Nature* 1992; **358**: 15-16

94 **Lowe SW**, Ruley HE. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev* 1993; **7**: 535-545

95 **Scheffner M**, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 1993; **75**: 495-505

96 **Park US**, Park SK, Lee YI, Park JG, Lee YI. Hepatitis B virus-X protein upregulates the expression of p21waf1/cip1 and prolongs G1-->S transition via a p53-independent pathway in

human hepatoma cells. *Oncogene* 2000; **19**: 3384-3394
97 **Chen X**, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* 1996; **10**: 2438-2451
98 **Han J**, Yoo HY, Choi BH, Rho HM. Selective transcriptional regulations in the human liver cell by hepatitis B viral X protein. *Biochem Biophys Res Commun* 2000; **272**: 525-530
99 **Livezey KW**, Negorev D, Simon D. Hepatitis B virus-transfected Hep G2 cells demonstrate genetic alterations and de novo viral integration in cells replicating HBV. *Mutat Res* 2000; **452**: 163-178
100 **Kobayashi S**, Matsushita K, Saigo K, Urashima T, Asano T, Hayashi H, Ochiai T. P21WAF1/CIP1 messenger RNA expression in hepatitis B, C virus-infected human hepatocellular carcinoma tissues. *Cancer* 2001; **91**: 2096-2103
101 **Wu CG**, Salvay DM, Forgues M, Valerie K, Farnsworth J, Markin RS, Wang XW. Distinctive gene expression profiles associated with Hepatitis B virus x protein. *Oncogene* 2001; **20**: 3674-3682
102 **Haviv I**, Vaizel D, Shaul Y. The X protein of hepatitis B virus coactivates potent activation domains. *Mol Cell Biol* 1995; **15**: 1079-1085
103 **Akagi T**, Ono H, Shimotohno K. Expression of cell-cycle regulatory genes in HTLV-I infected T-cell lines: possible involvement of Tax1 in the altered expression of cyclin D2, p18Ink4 and p21Waf1/Cip1/Sdi1. *Oncogene* 1996; **12**: 1645-1652
104 **Cereseto A**, Diella F, Mulloy JC, Cara A, Michieli P, Grassmann R, Franchini G, Klotman ME. p53 functional impairment and high p21waf1/cip1 expression in human T-cell lymphotropic/leukemia virus type I-transformed T cells. *Blood* 1996; **88**: 1551-1560
105 **Mesnard JM**, Devaux C. Multiple control levels of cell proliferation by human T-cell leukemia virus type 1 Tax protein. *Virology* 1999; **257**: 277-284
106 **Faktor O**, Shaul Y. The identification of hepatitis B virus X gene responsive elements reveals functional similarity of X and HTLV-I tax. *Oncogene* 1990; **5**: 867-872
107 **Morozov A**, Shiyanov P, Barr E, Leiden JM, Raychaudhuri P. Accumulation of human papillomavirus type 16 E7 protein bypasses G1 arrest induced by serum deprivation and by the cell cycle inhibitor p21. *J Virol* 1997; **71**: 3451-3457
108 **Ruesch MN**, Laimins LA. Initiation of DNA synthesis by human papillomavirus E7 oncoproteins is resistant to p21-mediated inhibition of cyclin E-cdk2 activity. *J Virol* 1997; **71**: 5570-5578
109 **Butz K**, Geisen C, Ullmann A, Zentgraf H, Hoppe-Seyler F. Uncoupling of p21WAF1/CIP1/SDI1 mRNA and protein expression upon genotoxic stress. *Oncogene* 1998; **17**: 781-787
110 **Fiedler N**. Kombinierter Effekt des Hepatitis B Virus X Proteins und des Tumorsuppressorproteins p53 auf virale und zelluläre Promotoren [Diplomarbeit]. Gießen: Justus-Liebig-Universität, 1996
111 **Tarn C**, Lee S, Hu Y, Ashendel C, Andrisani OM. Hepatitis B virus X protein differentially activates RAS-RAF-MAPK and JNK pathways in X-transforming versus non-transforming AML12 hepatocytes. *J Biol Chem* 2001; **276**: 34671-34680

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Virological course of hepatitis A virus as determined by real time RT-PCR: Correlation with biochemical, immunological and genotypic profiles

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Abstract

AIM: To undertake analysis of hepatitis A viral load, alanine aminotransferase (ALT), and viral genotypes with duration of viremia, and to correlate these parameters with $CD4^+$/ $CD8^+$ lymphocyte populations that control cell-mediated immunity.

METHODS: Cell counts were carried out using fresh whole blood collected in EDTA vials using a fluorescence activated cell sorter. Hepatitis A virus (HAV) RNA was extracted from blood serum, reverse transcribed into cDNA and quantified by Real-Time polymerase chain reaction and was genotyped.

RESULTS: Among 11 patients, 10 could be analyzed completely. Of these, 3 had severe acute hepatitis (s-AH) and the remainder had a self-limited acute hepatitis A (AHA), with one patient with fulminant disease (encephalopathy Grade Ⅳ) dying on the 4th d. The ALT level was significantly higher both in AHA (1070.9 ± 894.3; P = 0.0014) and s-AH (1713.9 ± 886.3; P = 0.001) compared to normal controls (23.6 ± 7.2). The prothrombin time in s-AH patients (21.0 ± 2.0; P = 0.02) was significantly higher than in AHA (14.3 ± 1.1; P = 0.44). The $CD4^+/CD8^+$ ratio in AHA patients (1.17 ± 0.11; P = 0.22) and s-AH (0.83 ± 0.12; P = 0.0002) were lower than seen in normal healthy controls (1.52). Self-limited cases had peak viral load at the beginning of analysis while in s-AH patients this occurred at the 15th or 30th d. In acute and severe groups, one patient each belonged to genotype IA, with the remaining 8 cases belonging to genotype IIIA. The only fulminant hepatic failure case belonged to genotype IA. HAV viral load and ALT values collected during the entire course of the self-limited infection were directly correlated but this was not the case for s-AH patients.

CONCLUSION: Based on a small-scale study, the persistently higher viral load of s-AH might be due to diminished cellular immunity and hemolysis. The duration of viremia was dependent on the host, as the viral genotype had no apparent role in clinical outcome of AVH and s-AH cases.

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Key words: Viral load; Real-time PCR; Immunological response; Severe acute hepatitis; Self-limited acute hepatitis

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INTRODUCTION

Hepatitis A virus (HAV) is a common cause of acute viral infections in humans[1]. It is a ubiquitous virus readily transmitted by the feco-oral route[2-5]. Nucleotide sequence analysis of HAV has classified the virus in seven different genotypes, which include human (Ⅰ, Ⅱ, Ⅲ, and Ⅶ) and simian (Ⅳ, Ⅴ, and Ⅵ) genotypes[6]. HAV infection follows a benign course; it is often asymptomatic in younger children, but can develop into a fatal fulminant form or severe acute hepatitis in older persons[7-9].

To date, however, there is limited knowledge of viral load, or the length of viral persistence both in the blood circulation and in fecal excretion. It has been reported that a relapse may occur 30-90 d after the initial onset of the disease[10-12] and virus has been detected in the stool of patients[13]. Recently, outbreaks of HAV have occurred among hemophiliacs receiving organic solvent and detergent-treated factor Ⅷ, a fact that stresses the potential usefulness of a reliable and widely applicable technique for quantifying viral load in blood samples[14-18]. The level and the length of HAV viremia involve the additional risk of the carrier becoming an infectious source of hepatitis A[19].

We undertook further examination of hepatitis A viremia during the course of infection to understand whether viral load was correlated with cell-mediated immunity. The pathogenetic mechanisms underlying hepatocellular injury in acute hepatitis are poorly understood[20]. There is general agreement that HAV infection does not evolve to chronic hepatitis in man[21], and immune mechanisms have been suspected of playing a major role in eliminating virus-infected liver cells[22-24]. The aim of this study was to undertake analysis of HAV viral load, alanine aminotransferase, and viral genotypes with the duration of viremia, and to correlate these parameters with populations of $CD4^+/CD8^+$ lymphocytes that controls cell-mediated immunity.

MATERIALS AND METHODS

Patients and blood samples

Patients attending the Medical Out Patient Department of Lok Nayak Hospital, New Delhi, with the characteristic symptoms of acute viral hepatitis such as jaundice, fever, general malaise, fatigue, nausea, vomiting, anorexia and right upper quadrant discomfort, were screened for the study. Ten mL of blood were collected by venipuncture from those patients, who gave consent for five different visits. The study was approved by the ethical committee of Maulana Azad Medical College, as per the Declaration of Helsinki (1995). Consecutive blood samples were collected from 10 acute hepatitis A patients on the 0th, 7th, 15th, 30th and 45th d between July 2004 and June 2005. No sample was collected before the onset of symptoms. The '0th' d was defined as the first day when the patient presented after the onset of jaundice. Ten healthy subjects who had no evidence of liver disease or dysfunction were taken as control.

Serological tests

Laboratory examination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), and total bilirubin levels were carried out by standard methods. IgM anti-HAV were detected by ELISA (HAVAB-MEIA); Abbott Laboratories, North Chicago, IL), IgM anti-HEV (Qiagen, Hilden, Germany), HBsAg (Qiagen, Hilden, Germany) and anti-HCV (Bio-Rad, San Francisco, CA, USA) were measured according to the manufacturer's protocol.

FACS analysis of T-lymphocyte profile

One milliliter whole blood was collected into a vial containing EDTA and was employed for $CD4^+$ (T helper) and $CD8^+$ (T suppressor) cell counts within 24 h of collection, using a Fluorescence Activated Cell Sorter (FACS) (Becton Dickenson Electronics Laboratory, Mountain View, California). This system quantifies $CD4^+$, $CD8^+$ and $CD3^+$ T lymphocytes as absolute numbers of lymphocytes per μL (mm^3) of blood, and the $CD4^+/CD8^+$ T lymphocyte ratio. Samples from healthy controls and patients were also run for cell counts using the manufacturer's protocol and reagents.

Primers, probe and standard for real-time amplification

HAV RNA was extracted using the QIAamp™ viral RNA extraction kit (Qiagen, Germany). Viral RNA was amplified using primers derived from the most constant region, the 5' non-coding region (5' NCR)[25,26]. The primers used were, forward primer HAV1 (22: 5'-TTT CCG GAG CCC CTC TTG-3'), as wild type (M14707) reverse primers HAV2 (85: 5'-AAA GGG AAA TTT AGC CTA TAG CC-3') and HAV3 (85: 5'-AAA GGG AAA ATT TAG CCT ATA GCC-3'), and HAV-probe (58: 5' -FAM-ACT TGA TAC CTC ACC GCC GTT TGC CT-TAMRA-3') and RNA standard representing the 5'NCR region was constructed according to Costa-Mattioli *et al*[27].

Fluorogenic quantitative Real-Time PCR and direct sequencing

RT-PCR was carried out with a HAV quantification kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The total volume of the reaction mixture was 25 μL (15 μL of mastermix with 10 μL of the RNA template) in 0.2 mL tubes. The capillaries were sealed, centrifuged, and transferred to the Rotor Gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). Reverse-transcription was done for 15 min at 50℃ followed by 5 min denaturation at 95℃. The corresponding cDNA's were amplified by PCR (20 s at 95℃, 30 s at 50℃ acquiring FAM, and 20 s at 72℃) over 45 cycles, and an 87 bp fragment was obtained. The CT values from the clinical samples were plotted on the standard curve, and the number of copies was calculated automatically.

PCR amplification from part of the VP1/2A region of HAV genome was directly sequenced for genotyping[28-30]. Sequencing was done with an ABI Prism 310 Genetic Analyzer (ABI, Foster City, CA). Sequencing analysis was performed using ClustalW and the phylogenetic inference by version 1.81 of the PHYLIP software package (Professor J. Felsenstein, Department of Genetics, University of Washington, Washington, DC).

Statistical analysis

All data were analyzed by two tailed tests, and a *P* value less than 0.05 was considered significant. We used chi-square test and student's t-test as appropriate.

RESULTS

Comparison of clinical features between AVH and s-AH groups

The average age of all patients was 20.8 ± 15.5 years

Table 1 Clinical, biochemical and immunological characterization of different groups (AVH, s-AH, and normal control)

Characteristic	AVH[1]	s-AH[2]	Normal Control[3]
No. of cases	07	03	10
Sex (M/F)	3/4	3/0	7/3
Age (mean ± SD)	0.4 ± 17.9	27.7 ± 2.08	26.2 ± 3.6
ALT (mean ± SD; IU/L)	1070.9 ± 894.3	1713.9 ± 886.3	23.6 ± 7.2
AST (mean ± SD; IU/L)	621.3 ± 242.8	1614 ± 234.7	23.8 ± 5.8
T. Bilirubin (mean ± SD; mg/dL)	9.8 ± 3.8	24.5 ± 3.0	0.74 ± 0.43
Prothrombin Time (Seconds)	14.3 ± 1.1	21.0 ± 2.0	12.5 ± 0.5
Mean $CD4^+/CD8^+$	1.17 ± 0.11	0.83 ± 0.12	1.52 ± 0.11

ALT: AVH *vs* s-AH $P = 0.29$ (not significant); AVH *vs* NC $P = 0.0014$ (highly significant); s-AH *vs* NC $P = 0.001$ (highly significant); **AST:** AVH *vs* s-AH $P = 0.0034$ (highly significant); AVH *vs* NC $P = 0.00014$ (highly significant); s-AH *vs* NC $P = 0.00044$ (highly significant); **T. Bil:** AVH *vs* s-AH $P = 0.076$ (significant); AVH *vs* NC $P = 0.00017$ (highly significant); s-AH *vs* NC $P = 0.0023$ (highly significant); **PT:** AVH *vs* s-AH $P = 0.11$ (not significant); AVH *vs* NC $P = 0.44$ (not significant); s-AH *vs* NC $P = 0.02$ (significant); **Mean $CD4^+/CD8^+$:** AVH *vs* s-AH $P = 0.46$ (not significant); AVH *vs* NC $P = 0.22$ (not significant); s-AH *vs* NC $P = 0.0002$ (highly significant). **Note**: AVH[1] (Acute viral hepatitis); s-AH[2] (Severe acute hepatitis); NC[3] (Normal Control).

and ranged from 3 to 59 years. The average age and sex ratio of two groups of patients is shown in Table 1. 10 of 11 patients could be followed completely, of which 3 progressed to severe acute hepatitis (s-AH), which is defined on the basis of a prothrombin time (PT) < 40% of normal range. The remainder of patients had self-limited acute hepatitis A (AHA) and one died of fulminant hepatic failure (Grade Ⅳ encephalopathy) at the 4th d of follow up. There was no difference between the groups with respect to clinical symptoms, such as flu-like prodromes (including arthralgia, or headache), fever, nausea, vomiting, abdominal pain, pruritus, and diarrhea.

Comparison of biochemical features between AVH and s-AH groups

In Figure 1, the time course of viral load and serum transaminase ALT levels is presented for all patients. ALT values for both groups followed a decreasing trend towards normal from the initial to final day of follow up. The mean liver function profile of s-AH patients was higher compared to the AVH cases as shown in Table 1. The mean prothrombin time (PT) of s-AH patients was (21.0 ± 2.0; $P = 0.02$) significantly higher than that of acute cases (14.3 ± 1.1; $P = 0.44$).

Comparison of immunological profiles between AVH and s-AH groups

The mean immunological ($CD4^+$/ $CD8^+$) ratio in patients with acute viral hepatitis A was (1.17 ± 0.1) higher than that in the severe acute cases (0.83 ± 0.12). As shown in Table 1, the $CD4^+/CD8^+$ ratio in normal controls (NC) (1.52 ± 0.11) ratio was almost twice as high as in s-AH ($P = 0.0002$). There was no significant decrease in the immunological ratio of AVH cases compared to normal controls.

Comparison of viral load between AVH and s-AH groups

In the acute case, Ind-301, the viral load on the initial day was 4.5×10^5, and decreased further to > 10^3 copies/mL. As shown in Figure 1, Ind-303 and Ind-306 followed almost similar trends with viral loads of 2.6×10^5 and 1.2×10^5 respectively at 0th d while < 10^2 copies/mL still persisted at the end of follow up. The viral load of Ind-304 was 1.0×10^5 at start and complete elimination of the virus with zero copies/mL was seen at the end of follow up, as shown in Figure 1. Patients Ind-308 and Ind-310 displayed a peak viral load at 0th d like the cases described above, while Ind-309 peaked on the 15th d, but values then decreased to less than 100 copies/mL in all three. In the severe cases Ind-302 and Ind-305, the viral load on the initial day of follow up was 1.2×10^5 and 1.1×10^5 respectively, and reached a peak on the 30th d, quite different from the acute case shown in Figure 1. At the end of follow up, the viral load was significantly higher (> 1.0×10^5) as compared to AVH. In-patient Ind-307, viral load reached the peak (4.6×10^5) at 15th d and decreased subsequently to 4.3×10^4 copies/mL at the end of the follow up.

Comparison of genotype (s) between AVH and s-AH groups

In Table 2, the maximal viral load was compared with genotyping and geographical distribution. As shown in phylogenetic tree (Figure 2), the patients Ind-301 (DQ179131) and Ind-302 (DQ179132) were categorized as genotype IA, while the remainder Ind-303 (DQ179133), Ind-304 (DQ179134), Ind-305 (DQ179135), Ind-306 (DQ179136), Ind-307 (DQ179134), Ind-308 (DQ182495), Ind-309 (DQ182496), and Ind-310 (DQ182497)) were classified as the ⅢA genotype. The only fulminant hepatic failure case, Ind-274 (DQ182500), belonged to genotype ⅠA.

DISCUSSION

Hepatitis A remains the most frequent form of viral hepatitis observed in a large number of countries[31,32]. Recent publications have demonstrated that the duration of the viremic phase is much longer than assumed[10,27]. A serum HAV viral load assay could therefore be helpful in the management of severe hepatitis A. Real-Time reverse transcription (Rotor Gene 3000, Corbett Research, Sydney, Australia), was used for the quantitative detection of the HAV genome in human sera in individuals who displayed varying disease courses[27,33,34]. The fluorescence signal due to the cleavage of the fluorogenic probe is generated only if the target sequence for the probe is amplified by the PCR. Therefore, no signals are generated by non-specific amplification.

The alanine aminotransferase level of AVH cases on the initial day was significantly higher (⩾ 10^3) and the decreased upon subsequent follow up, which corresponds to earlier findings that demonstrate a direct correlation of viral load with serum ALT[27,35]. In our study, the mean prothrombin time in AVH was not higher while severe cases showed significant elevations compared to normal controls. This could be due to anemia (hemolysis) as this

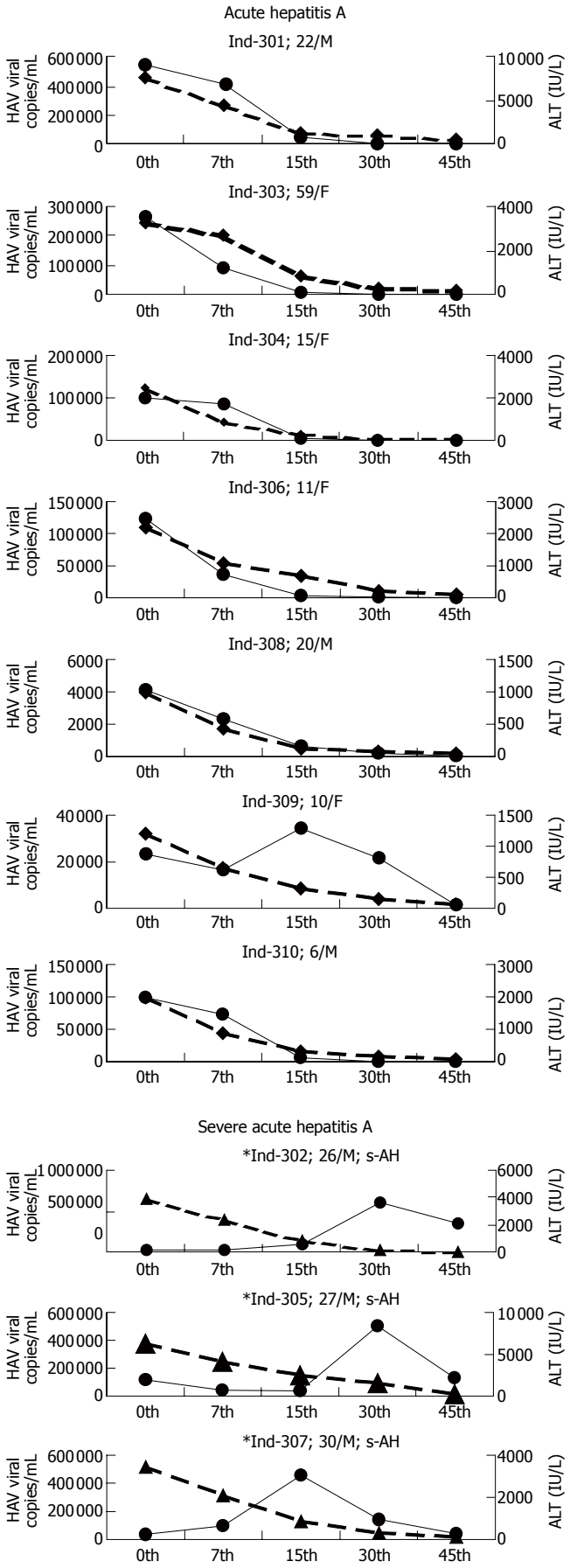

Figure 1 Relationship of hepatitis A virus (HAV) viral load (●) to levels of alanine aminotransferase (ALT) (▲) in serum at different day of follow up.

Table 2 Comparison of patient's genotypes to viral load and geographical distribution

Patients	Genotype	Maximal viral load Copies/mL	Geographical distribution
Ind-301	I A	4.5×10^5	New Delhi
[1]Ind-302	I A	6.0×10^5	New Delhi
Ind-303	III A	2.6×10^5	Uttar Pradesh
Ind-304	III A	1.0×10^5	Delhi
[1]Ind-305	III A	5.0×10^5	Delhi
Ind-306	III A	1.2×10^5	New Delhi
[1]Ind-307	III A	4.6×10^5	Uttar Pradesh
Ind-308	III A	4.1×10^3	New Delhi
Ind-309	III A	3.4×10^4	Haryana
Ind-310	III A	9.8×10^4	New Delhi

[1]Represents severe acute hepatitis.

is not uncommon during viral hepatitis[36]. We excluded glucose-6 phosphate dehydrogenase (G6PD) deficiency as a cause of hemolysis and anemia in our cases[36,37].

The immunological changes in acute cases was not significantly different from those in severe hepatitis A. The lymphocyte ratio (mean $CD4^+/CD8^+$) of the severe patients was, however, significantly lower, which could be due to diminished cellular immunity as compared to the normal controls. Previous studies have also shown that generation of $CD4^+$ T cells in the thymus is severely impeded as either a direct or indirect consequence of active viral replication[35,38]. We speculate that severe HAV infection may be triggered by diminished cellular immunity in susceptible patients, which may have increased the liver damage due to hepatitis A.

On the basis of our results, genome quantities measured on the first (0th) d of clinical diagnosis in HAV infected humans reached peaked copies/mL in acute viral hepatitis and attained normality towards the end of follow up. The kinetics of peak viral load attainment in s-AH was quite different from that in the acute self-limited cases since at the end of follow up high copies/mL still persisted, as shown in Figure 1. The acute results confirm the levels recently estimated by Chudy *et al*[15]. The progression of severity due to diminished cellular immunity and hemolysis might be directly linked to high viral persistence throughout the follow up.

Most of the patients examined during acute self-limited illness belonged to genotype III A, other than Ind-301 who belonged to I A[6]. Among the severe cases, Ind-302 belonged to I A while other two belonged to III A, which means genotypic variations likely do not play a crucial role in determination of the viral load as described earlier by Normann[35]. The only FHF case, who died at the 4th d of follow up, belonged to genotype I A. The question arises whether the duration of viremia is dependent on the genotype, or the immunological and/or biochemical profile. Our results showed that the duration of the viremia was dependent on the host, as the viral genotype had no role in acute self-limited illness and severe acute hepatitis A cases. This differs considerably from earlier

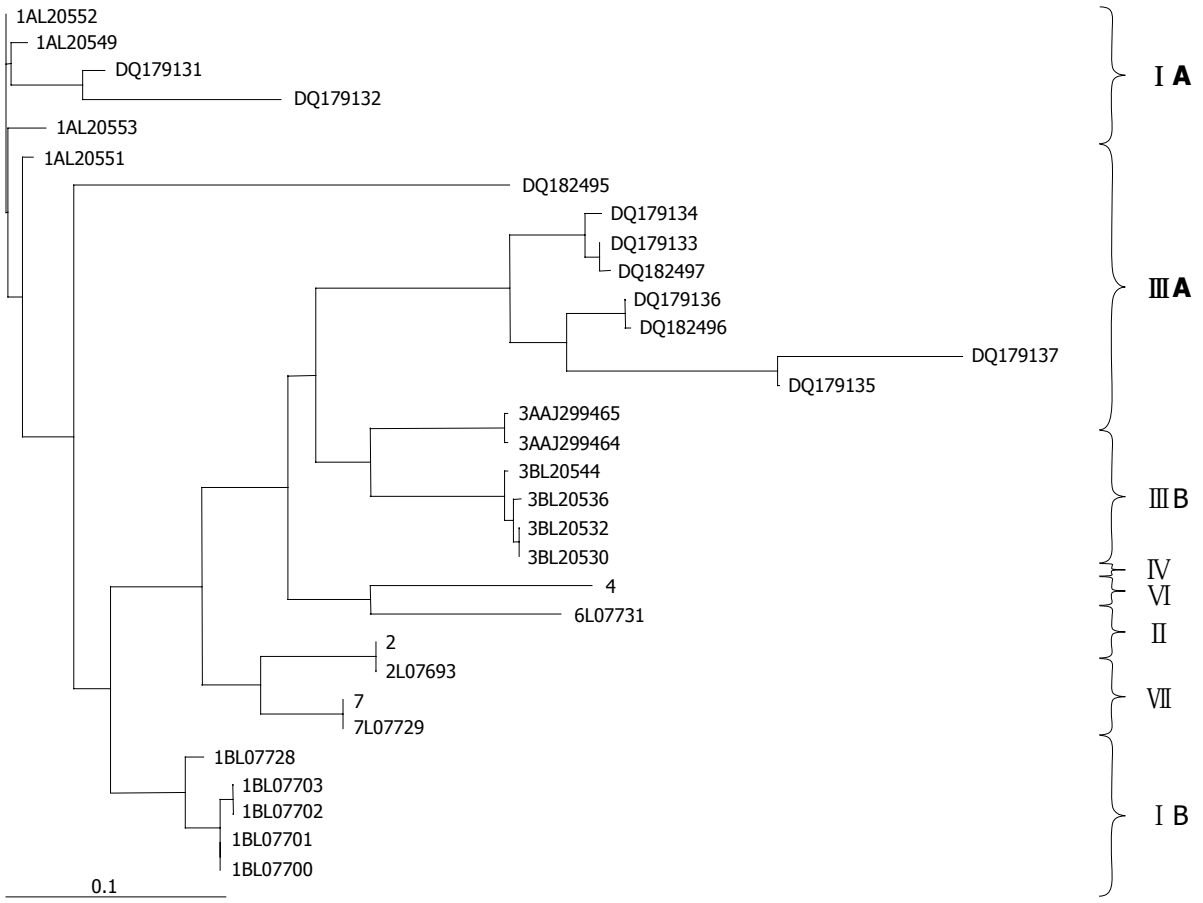

Figure 2 A neighbor-joining phylogenetic tree for isolates of hepatitis A virus based on the sequencing of the VP1-P2A region. Isolates DQ179131 (Ind-301), DQ179132 (Ind-302), DQ179133 (Ind-303), DQ179134 (Ind-304), DQ179135 (Ind-305), DQ179136 (Ind-306), DQ179137 (Ind-307), DQ182495 (Ind-308), DQ182496 (Ind-309), DQ182497 (Ind-310) were collected during the present study, in PCR Hepatitis Laboratory, MAM College and associated LNJ Hospital, New Delhi, India.

findings which showed that a long duration of viremia was found in patients infected with HAV genotype IA[35].

In conclusion, HAV viral load and alanine aminotransferase (ALT) values collected during the entire course of a self-limited acute infection were directly correlated, but this was not found in s-AH cases. The duration of viremia was dependent on the host (biochemical and immunological profiles), as the viral genotype had no role in the various groups studied. The mean prothrombin time in severe acute hepatitis was higher than seen in acute self-limited illness. The immunological ($CD4^+/CD8^+$) ratio of s-AH was quite low compared to the acute self-limited illness, and s-AH patients showed diminished cellular immunity and complications. There was no difference in the final clinical outcome and recovery of liver function was seen in all patients. The limitation of the study is that the number of patients examined was relatively small. Therefore, there is a need for further research on the duration and magnitude of HAV viremia in a large cohort of human patients to properly document complications and management.

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REFERENCES

1 **Centers for Disease Control and Prevention**. Hepatitis surveillance report No.56. Atlanta GA: Centers for Disease control, 1995

2 **Sjogren MH**. Hepatitis A. In: Schiff ER, Sorrell MF, Maddery WC, editors. Schiff's Diseases of the Liver. 8th ed. Philadelphia: Lippincott-Raven, 1999: 745-756

3 **Hadler SC**. Global impact of hepatitis A virus infection changing patterns. In: Hollinger FB, Lemon SM, Margolis HS, editors. Viral hepatitis and liver disease. Baltimore, MD: Williams & Wilkins, 1991: 14-20

4 **Bölke E**, Flehmig B. New epidemiological patterns of hepatitis A and B infections in Germany. *Zentralbl Hyg Umweltmed* 1995; **196**: 511-514

5 **Niu MT**, Polish LB, Robertson BH, Khanna BK, Woodruff BA, Shapiro CN, Miller MA, Smith JD, Gedrose JK, Alter MJ. Multistate outbreak of hepatitis A associated with frozen strawberries. *J Infect Dis* 1992; **166**: 518-524

6 **Robertson BH**, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, Widell A, Margolis HS, Isomura S, Ito K. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J Gen Virol* 1992; **73** (Pt 6): 1365-1377

7 **Takahashi Y**, Okuda K. Fulminant and subfulminant hepatitis in Japan--etiological considerations. *Indian J Gastroenterol* 1993;

12 Suppl 3: 19-21
8 **Vento S**, Garofano T, Renzini C, Cainelli F, Casali F, Ghironzi G, Ferraro T, Concia E. Fulminant hepatitis associated with hepatitis A virus superinfection in patients with chronic hepatitis C. *N Engl J Med* 1998; **338**: 286-290
9 **Sainokami S**, Abe K, Ishikawa K, Suzuki K. Influence of load of hepatitis A virus on disease severity and its relationship with clinical manifestations in patients with hepatitis A. *J Gastroenterol Hepatol* 2005; **20**: 1165-1175
10 **Bower WA**, Nainan OV, Han X, Margolis HS. Duration of viremia in hepatitis A virus infection. *J Infect Dis* 2000; **182**: 12-17
11 **Yotsuyanagi H**, Iino S, Koike K, Yasuda K, Hino K, Kurokawa K. Duration of viremia in human hepatitis A viral infection as determined by polymerase chain reaction. *J Med Virol* 1993; **40**: 35-38
12 **Fujiwara K**, Yokosuka O, Ehata T, Imazeki F, Saisho H, Miki M, Omata M. Frequent detection of hepatitis A viral RNA in serum during the early convalescent phase of acute hepatitis A. *Hepatology* 1997; **26**: 1634-1639
13 **Sjogren MH**, Tanno H, Fay O, Sileoni S, Cohen BD, Burke DS, Feighny RJ. Hepatitis A virus in stool during clinical relapse. *Ann Intern Med* 1987; **106**: 221-226
14 **Kedda MA**, Kew MC, Cohn RJ, Field SP, Schwyzer R, Song E, Fernandes-Costa F. An outbreak of hepatitis A among South African patients with hemophilia: evidence implicating contaminated factor VIII concentrate as the source. *Hepatology* 1995; **22**: 1363-1367
15 **Chudy M**, Budek I, Keller-Stanislawski B, McCaustland KA, Neidhold S, Robertson BH, Nübling CM, Seitz R, Löwer J. A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. *J Med Virol* 1999; **57**: 91-99
16 **Corey L**, Holmes KK. Sexual transmission of hepatitis A in homosexual men: incidence and mechanism. *N Engl J Med* 1980; **302**: 435-438
17 **Mannucci PM**. Outbreak of hepatitis A among Italian patients with haemophilia. *Lancet* 1992; **339**: 819
18 **Normann A**, Graff J, Gerritzen A, Brackmann HH, Flehmig B. Detection of hepatitis A virus RNA in commercially available factor VIII preparation. *Lancet* 1992; **340**: 1232-1233
19 **Inoue K**, Yoshiba M, Yotsuyanagi H, Otsuka T, Sekiyama K, Fujita R. Chronic hepatitis A with persistent viral replication. *J Med Virol* 1996; **50**: 322-324
20 **Kurane I**, Binn LN, Bancroft WH, Ennis FA. Human lymphocyte responses to hepatitis A virus-infected cells: interferon production and lysis of infected cells. *J Immunol* 1985; **135**: 2140-2144
21 **Vallbracht A**, Gabriel P, Zahn J, Flehmig B. Hepatitis A virus infection and the interferon system. *J Infect Dis* 1985; **152**: 211-213
22 **Vallbracht A**, Gabriel P, Maier K, Hartmann F, Steinhardt HJ, Müller C, Wolf A, Manncke KH, Flehmig B. Cell-mediated cytotoxicity in hepatitis A virus infection. *Hepatology* 1986; **6**: 1308-1314
23 **Vallbracht A**, Maier K, Stierhof YD, Wiedmann KH, Flehmig B, Fleischer B. Liver-derived cytotoxic T cells in hepatitis A virus infection. *J Infect Dis* 1989; **160**: 209-217
24 **Stapleton JT**. Host immune response to hepatitis A virus. *J Infect Dis* 1995; **171** Suppl 1: S9-14
25 **Cohen JI**, Ticehurst JR, Purcell RH, Buckler-White A, Baroudy BM. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J Virol* 1987; **61**: 50-59
26 **Jansen RW**, Newbold JE, Lemon SM. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for in vitro replication. *Virology* 1988; **163**: 299-307
27 **Costa-Mattioli M**, Monpoeho S, Nicand E, Aleman MH, Billaudel S, Ferré V. Quantification and duration of viraemia during hepatitis A infection as determined by real-time RT-PCR. *J Viral Hepat* 2002; **9**: 101-106
28 **Hussain Z**, Das BC, Husain SA, Asim M, Chattopadhyay S, Malik A, Poovorawan Y, Theamboonlers A, Kar P. Hepatitis A viral genotypes and clinical relevance: Clinical and molecular characterization of hepatitis A virus isolates from northern India. *Hepatol Res* 2005; **32**: 16-24
29 **Theamboonlers A**, Jantaradsamee P, Chatchatee P, Chongsrisawat V, Mokmula M, Poovorawan Y. Molecular characterization of hepatitis-A-virus infections, in the context of two outbreaks in southern Thailand. *Ann Trop Med Parasitol* 2002; **96**: 727-734
30 **Najarian R**, Caput D, Gee W, Potter SJ, Renard A, Merryweather J, Van Nest G, Dina D. Primary structure and gene organization of human hepatitis A virus. *Proc Natl Acad Sci USA* 1985; **82**: 2627-2631
31 **Steffen R**. Risk of hepatitis A in travellers. *Vaccine* 1992; **10** Suppl 1: S69-S72
32 **Flehmig B**, Normann A, Bohnen D. Transmission of hepatitis A virus infection despite vaccination. *N Engl J Med* 2000; **343**: 301-302
33 **Monpoeho S**, Dehée A, Mignotte B, Schwartzbrod L, Marechal V, Nicolas JC, Billaudel S, Férré V. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* 2000; **29**: 88-93
34 **Arnal C**, Ferre-Aubineau V, Mignotte B, Imbert-Marcille BM, Billaudel S. Quantification of hepatitis A virus in shellfish by competitive reverse transcription-PCR with coextraction of standard RNA. *Appl Environ Microbiol* 1999; **65**: 322-326
35 **Normann A**, Jung C, Vallbracht A, Flehmig B. Time course of hepatitis A viremia and viral load in the blood of human hepatitis A patients. *J Med Virol* 2004; **72**: 10-16
36 **Lyons DJ**, Gilvarry JM, Fielding JF. Severe haemolysis associated with hepatitis A and normal glucose-6-phosphate dehydrogenase status. *Gut* 1990; **31**: 838-839
37 **Raffensperger EC**. Acute acquired hemolytic anemia in association with acute viral hepatitis. *Ann Intern Med* 1958; **48**: 1243-1253
38 **Ajdukiewicz AB**, Fox RA, Dudley FJ, Doniach D, Sherlock S. Immunological studies in an epidemic of infective, short-incubation hepatitis. *Lancet* 1972; **1**: 803-805

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H pylori receptor MHC class II contributes to the dynamic gastric epithelial apoptotic response

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Abstract

AIM: To investigate the role of MHC class Ⅱ in the modulation of gastric epithelial cell apoptosis induced by *H pylori* infection.

METHODS: After stimulating a human gastric epithelial cell line with bacteria or agonist antibodies specific for MHC class Ⅱ and CD95, the quantitation of apoptotic and anti-apoptotic events, including caspase activation, BCL-2 activation, and FADD recruitment, was performed with a fluorometric assay, a cytometric bead array, and confocal microscopy, respectively.

RESULTS: Pretreatment of N87 cells with the anti-MHC class Ⅱ IgM antibody RFD1 resulted in a reduction in global caspase activation at 24 h of *H pylori* infection. When caspase 3 activation was specifically measured, crosslinking of MHC class Ⅱ resulted in markedly reduced caspase activation, while simple ligation of MHC class Ⅱ did not. Crosslinking of MHC class Ⅱ also resulted in an increased activation of the anti-apoptosis molecule BCL-2 compared to simple ligation. Confocal microscope analysis demonstrated that the pretreatment of gastric epithelial cells with a crosslinking anti-MHC class Ⅱ IgM blocked the recruitment of FADD to the cell surface.

CONCLUSION: The ability of MHC class Ⅱ to modulate gastric epithelial apoptosis is at least partially dependent on its crosslinking. The crosslinking of this molecule has anti-apoptotic effects during the earlier time points of *H pylori* infection. This effect is possibly mediated by the ability of MHC class Ⅱ to modulate the activation of the pro-apoptotic receptor Fas by blocking the recruitment of the accessory molecule FADD, and this delay in apoptosis induction could allow for prolonged cytokine secretion by *H pylori*-infected gastric epithelial cells.

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Key words: *H pylori*; Epithelium; Apoptosis; Class Ⅱ MHC

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INTRODUCTION

H pylori infects over half of the people in the world. Seropositivity may reach 80%-100% in underdeveloped nations. This gram negative bacterium is a major contributor to chronic gastritis and peptic ulcer formation, and is strongly associated with gastric carcinoma and lymphoma[1,2]. Gastric carcinoma remains the second most deadly form of cancer[3]. While much is known about the clinical manifestations of *H pylori* infection, how this pathogen manipulates gastric epithelial cells in the host to its advantage are unknown. Previous reports by our group have demonstrated that MHC class Ⅱ expressed on the surface of gastric epithelial cells serve as a receptor for *H pylori*[4,5]. A potential consequence of bacterial interaction with MHC class Ⅱ proteins is the subsequent crosslinking of these molecules. This may impact cellular responses key to the initiation and propagation of *H pylori* pathogenesis that results in tissue damage of the gastro-duodenal mucosa.

One such clinically significant cellular response to *H pylori* infection is apoptosis. The induction of apoptosis in MHC class Ⅱ$^+$ host cells able to direct the immune response would represent a mechanism by which the bacteria could impair local antigen presentation to T cells. Furthermore, induction of apoptosis would cause "leakiness" of the epithelium, leading to inflammation that could upregulate the expression of *H pylori* receptors on surrounding cells. For example, IFNγ, an inflammatory cytokine produced by CD4$^+$ T cells within the infected gastric mucosa, upregulates class Ⅱ MHC expression in gastric epithelial cells. However, uncontrolled epithelial apoptosis would

quickly lead to the destruction of *H pylori*'s niche within the gastric mucosa. Thus, mechanisms by which these bacteria could moderate the host apoptotic response must also be considered.

Previous reports show that CD95 (Fas) plays an important role in *H pylori*-mediated apoptosis of the gastric epithelium[6,7]. Once Fas is activated on the cell surface, the FADD (Fas associated death domain) protein is recruited to the cytoplasmic domains of the trimerized Fas on the plasma membrane. FADD is then responsible for the activation of caspase 8. However, the interaction between *H pylori* receptors and pro-apoptotic death receptors such as Fas has not been well investigated. Combined with our previous data demonstrating the role of MHC class Ⅱ in *H pylori* binding to gastric epithelial cells (GEC), it might be suggested that the complex dynamics regulating apoptosis during infection might be due to either complementary or antagonistic interactions between multiple signaling receptors on the cell surface. Furthermore, the possibility that MHC class Ⅱ crosslinking modulates pro-death accessory molecules within the cytoplasm must also be investigated.

MATERIALS AND METHODS

Cell and Bacterial Culture

The human gastric epithelial cell line N87 was obtained from ATCC and cultured in RPMI containing 100 mL/L fetal calf serum and supplemented with glutamine. *H pylori* cag+ clinical isolate LC-11[8] was grown on blood agar base (Becton Dickinson) at 37℃ under microaerobic conditions and harvested into Brucella broth containing 100 mL/L fetal bovine serum. Bacteria in broth were rocked gently overnight at 37℃ under microaerobic conditions prior to centrifugation. *H pylori* was resuspended in PBS and concentration was determined by absorbance at 530 nm using a spectrophotometer (1 A = 2×10^8 cfu/mL) (DU-65 Becton Dickinson Instruments, Fullerton, CA).

Antibodies

Monoclonal anti-human MHC class Ⅱ IgM (clone RFD1) was obtained from Serotec, Raleigh, NC. Monoclonal IgM antibody against CD-95 (clone IPO-4) used to induce apoptosis was obtained from Kamiya Biomedial Co., Seattle, WA. The hybridomas secreting anti-human MHC class Ⅱ IVA-12 and L243 (mIgG) were obtained from ATCC and were used to produce ascites fluid in mice and the antibodies were purified with a protein G column. Anti-human CD95-PE was obtained from Becton Dickinson/Pharmingen, San Jose, CA. Alexa-conjugated secondary antibodies were obtained from Molecular Probes Inc., Eugene, OR.

Global Caspase Activation Assay

The global (non-specific) activation of caspases in our cell line was quantified using the Homogeneous Caspase Activation kit from Roche Applied Science, Indianapolis, IN. Cells were grown in serum containing media in 96-well plates at a seeding density of 10^4 cells/well for 18 h prior to treatment. After treatment, the media was aspirated and a substrate-containing lysis solution was applied to the cells. After a 2 h incubation at 37℃, the cleaved substrate product resulting from the action of activated caspases was quantitated using a fluoremeter (λ = 521 nm).

Caspase 3 and BCL-2 activation

Caspase 3 activation and BCL-2 expression were quantitated using the Human Apoptosis Cytometric Bead Array kit from Becton Dickinson, Franklin Lakes, NJ. The experiments were conducted according to the kit instructions. Briefly, bead populations with distinct fluorescent intensities were coated with antibodies specific for activated caspase 3 and BCL-2. The capture beads, sample lysates, and PE-conjugated detection reagent were incubated together to form sandwich complexes. After washing, the beads were run through a flow cytometer to generate MFI data, which was then analyzed with Becton Dickinson CBA Analysis Software. Sample data was normalized with specific protein standards to provide quantification of the proteins of interest.

Confocal microscopy

Cells were grown on Collagen I-coated tissue culture inserts (BD Biosciences) to 50%-75% confluency. After cell permeabilization and intracellular staining, the inserts were mounted on glass slides with coverslips. Images were obtained on a Zeiss LSM510 META advanced laser scanning confocal microscope (LSCM). Approximately 50 separate images were obtained at 0.5-0.6 micron increments for X-Z axis reconstruction.

Statistical analysis

Data is presented as the mean ± SE and analyzed using Student's *t* test. Significance is defined as $P < 0.05$.

RESULTS

Global Caspase Activation Assay

To determine the effect of MHC class Ⅱ crosslinking at the cell surface on the activation of caspases, we cultured the gastric epithelial cell line N87 in the presence of 100 kU/L IFNγ for 48 h to induce the upregulation of surface MHC class Ⅱ. Cells were then rested in serum containing medium alone for 2 d prior to bacterial and antibody treatment. Cells pretreated with antibody were exposed to RFD1 (10 mg/L) for 30 min prior to the addition of *H pylori*. Camptothecin (10 μmd/L) was used as a positive control for caspase activation and an isotype antibody, which had no effect on the cells, was another control. After 24 h of infection, crosslinking MHC class Ⅱ prior to the addition of *H pylori* significantly ($P < 0.05$) reduced global caspase activation compared to *H pylori* infected samples with no pretreatment (Figure 1).

Apoptosis Cytometric Bead Array

Caspase 3 is an effector caspase whose activation is central in the apoptosis pathway triggered by Fas trimerization. To specifically examine the activation of caspase 3 as a result of MHC class Ⅱ ligation versus crosslinking, we used a cytometric bead array that incorporates antibodies

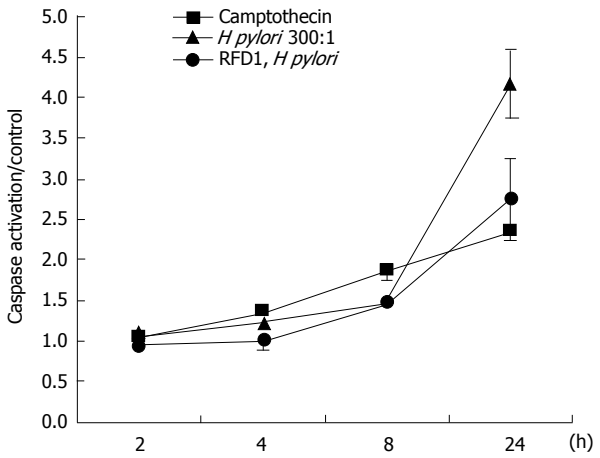

Figure 1 Total caspase activation detected using a colorometric assay. (means of the treatments / control (untreated) samples.

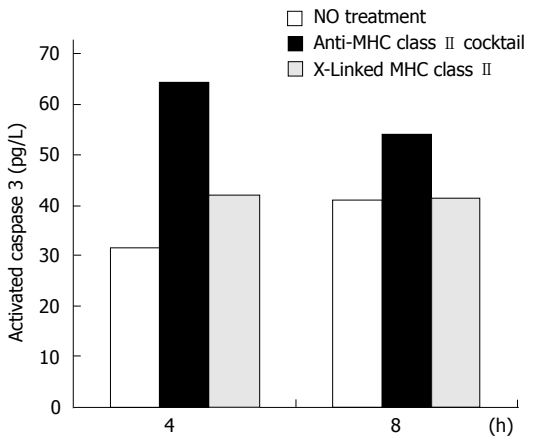

Figure 2 The effects of ligating *vs* crosslinking N87 cell surface MHC class II molecules on caspase 3 activation. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to activated caspase 3 standards to give values expressed as pg/mL of activated caspase 3.

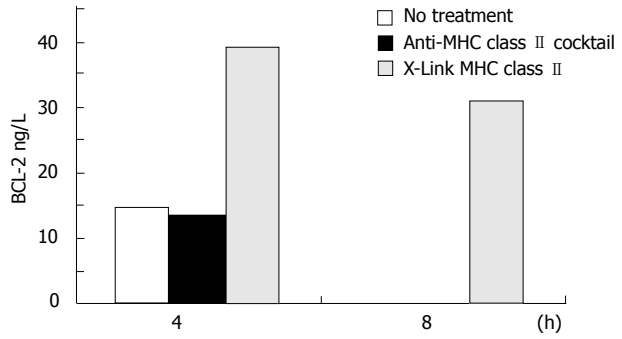

Figure 3 The effects of ligating *vs* crosslinking N87 cell surface MHC class II molecules on BCL-2 expression. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to BCL-2 standards to give values expressed as ng/L of BCL-2.

to the activated form of caspase 3. At 4 and 8 h treatment of IFNγ-treated N87 cells, crosslinking MHC class Ⅱ with biotinylated anti-MHC class Ⅱ IgG antibodies L243 and IVA12 resulted in a reduced activation of caspase 3 activation compared to ligating MHC class Ⅱ with unbiotinylated cocktail. After 4 h of treatment, ligation of MHC class Ⅱ resulted in a 103% increase in caspase 3 activation compared to a 33% increase after crosslinking. At 8 h of treatment, crosslinking MHC class Ⅱ resulted in a negligible increase in caspase 3 activation compared with control, while ligation with the antibody cocktail increased caspase 3 activation by 33% over control (untreated) samples (Figure 2).

The expression of the anti-apoptotic BCL-2 molecule was measured simultaneously with caspase 3 activation. As crosslinking of MHC class Ⅱ resulted in reduced activation of the pro-apoptotic caspase 3 compared to MHC class Ⅱ ligation, MHC class Ⅱ crosslinking but not ligation produced an increase in the expression of BCL-2. MHC class Ⅱ crosslinked samples showed a 169% increase over control in BCL-2 expression at 4 h treatment. After 8 h treatment, the MHC class Ⅱ crosslinked samples contained over 30 ng/L of BCL-2 while the untreated and MHC class Ⅱ ligated samples had no detectible BCL-2 (Figure 3).

Confocal microscopy analysis of FADD recruitment

Fas aggregation induces the recruitment of the adapter protein Fas-associated death domain (FADD) to the cytoplasmic tail of Fas. To determine whether MHC class Ⅱ crosslinking affects FADD recruitment, we did confocal microscopy in gastric epithelial cells treated with anti-Fas with and without pretreatment with anti-MHC class Ⅱ. N87 cells were treated with 100 kU/L IFNγ for 48 h to increase the surface expression of MHC class Ⅱ and Fas. Cells were then seeded onto filter inserts with media alone for 24 h before treatment. Prior to permeabilization, fixation, and staining, samples were A: left untreated (control), B: treated for 1 h with the anti-Fas IgM clone IPO-4 or C: pretreated with the anti-MHC class Ⅱ IgM clone RFD1 for 30 min prior to adding IPO-4. After washing, the cells were then permeabilized and fixed to allow staining of intracellular FADD. The filter inserts were then mounted onto slides and immediately visualized with a confocal microscope. The large square panel represents an X-Y axis "top-down" perspective on the adherent N87 cells. The top and side rectangular panels in each figure represent X-Z axis reconstructions, which provide an elevation view of the apical and basolateral sides of the cell section (Figure 4).

DISCUSSION

Apoptosis accounts for most of the cell loss in the gastrointestinal tract[9]. Because cellular turnover rate in the gut epithelium is so high, disturbances in the homeostasis of cell proliferation and cell death can potentially lead to disease states. If apoptosis were induced at a higher rate than new cell generation, tissue atrophy and ulceration might occur. Conversely, downregulation or blocking of apoptotic pathways might result in neoplasia. Furthermore, it is possible that hyperplasia is a response to pro-apoptotic stimuli or that increased apoptosis results from an induced hyperproliferative stimulus[10]. These potential scenarios, and the molecular dynamics responsible

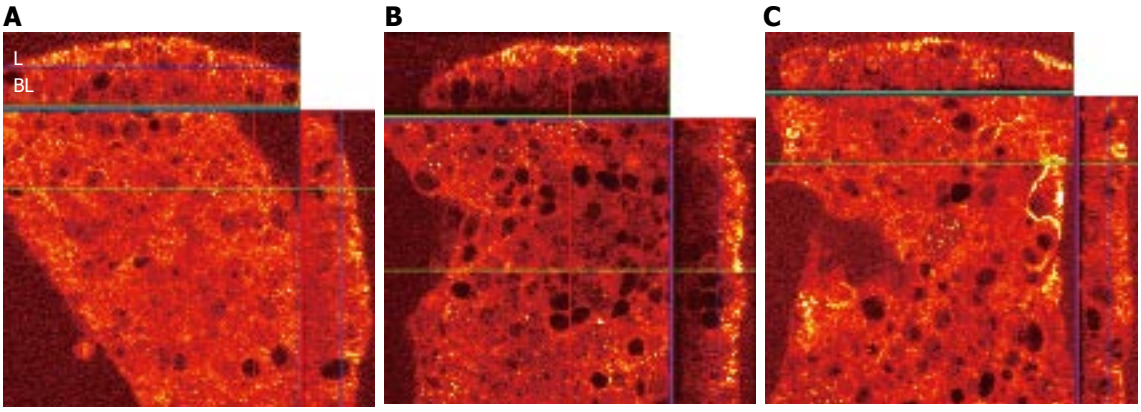

Figure 4 **A**: Viewing the X-Z panels, the FADD (stained red, with higher densities staining yellow) in untreated (control) cells distributed equally throughout the cytoplasm. "L" represents the apical (lumenal) surface and "BL" represents the basolateral surface of the N87 monolayer; **B**: Labeled FADD in cells treated with the Fas trimerizing antibody is recruited to the apical surface, leaving much of the cytoplasm with reduced FADD staining; **C**: When the anti-MHC class II IgM RFD1 is applied to the cell monolayer 30 min prior to treatment with the Fas agonist IPO4 IgM, the recruitment of FADD to the cell surface is markedly reduced.

for them, become particularly important when considering the divergent clinical manifestations of *H pylori* infection. Two clinical observations support the concept of mutually exclusive disease states resulting from a disturbance in the delicate balance between apoptosis and proliferation of the mucosal epithelium. The first stems from multiple studies that demonstrated patients exhibiting duodenal ulcers have a lower risk of developing gastric cancer[11,12]. The second suggests an inverse association between *H pylori* infection and esophageal adenocarcinoma[13,14]. Although these correlations continue to be scrutinized, clearly, any factor affecting the apoptotic levels within or causing the formation of ulcerative or neoplastic epithelial tissue is critical in influencing the ultimate disease state. Therefore, the divergent and somewhat mutually exclusive nature of *H pylori* disease pathology provides a strong incentive to elucidate the epithelial receptors for *H pylori* during infection, and to investigate results of receptor ligation and crosslinking on host cell apoptosis.

The apoptosis-inducing effect of *H pylori* on gastric epithelial cells has been thoroughly demonstrated[4-7,10,15-18]. Furthermore, our group's findings demonstrated that MHC class Ⅱ not only binds *H pylori*, but also can initiate signals affecting apoptotic pathways. It suggests an important role for this molecule in influencing *H pylori* pathogenesis. The literature offers conflicting reports on the apoptotic *vs* anti-apoptotic effects of MHC class Ⅱ binding. More specifically, there are opposing reports on the effect of MHC class Ⅱ ligation on the Fas death pathway in mouse and human B cells[19,20]. Previous studies from our group have revealed that signals induced by the long term (72 h) crosslinking of MHC class Ⅱ are pro-apoptotic[4,5]. However, the results we have reported here indicate that crosslinking of MHC class Ⅱ can induce anti-apoptotic effects at time points less than 24 h. Furthermore, we have demonstrated the ability of MHC class Ⅱ crosslinking to inhibit a key component of the Fas death pathway. Our findings that MHC class Ⅱ crosslinking inhibits *H pylori*-induced caspase activation, that ligation *vs* crosslinking of MHC class Ⅱ has differential effects on apoptotis-associated molecules, and that MHC class Ⅱ crosslinking inhibits FADD recruitment are intriguing because of their potential role in the divergent pathophysiologic host response to this human pathogen. By acting as a bacterial receptor, as well as modulating the important apoptotic processes during infection, MHC class Ⅱ becomes a critically important molecule in the context of *H pylori* pathogenesis. Nonetheless, it is important to continue to study all GEC surface molecules that have the potential to influence the signal events initiated by *H pylori* binding. There is significant evidence that other surface molecules in addition to MHC class Ⅱ are capable of binding *H pylori*; there have been numerous studies demonstrating the role of Lewis b (Le^b) blood group antigen in *H pylori* adherence to the epithelium[22,23]. More recently, it has been suggested that the dimeric form of the trefoil protein TFF1 avidly binds *H pylori*[24]. The implications of each of these findings in the physical interaction between bacterium and host is important because of the need to understand the molecules responsible for bacterial adhesion. They are also important because of the possibility that a particular bacterial receptor influences the downstream signal transduction events initiated when *H pylori* binds to a secondary receptor, or activates a death receptor.

Deciphering the complexity of *H pylori* pathogenesis in order to reduce its contribution to human gastric disease will require continued investigation into the interactions between bacterial receptors and their effects on the host epithelial cell homeostasis.

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REFERENCES

1 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991;

325: 1127-1131
2 **Correa P**. *Helicobacter pylori* and gastric carcinogenesis. *Am J Surg Pathol* 1995; **19** Suppl 1: S37-S43
3 **Crowe SE**. Helicobacter infection, chronic inflammation, and the development of malignancy. *Curr Opin Gastroenterol* 2005; **21**: 32-38
4 **Fan X**, Crowe SE, Behar S, Gunasena H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II major histocompatibility complex expression on adherence of *Helicobacter pylori* and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. *J Exp Med* 1998; **187**: 1659-1669
5 **Fan X**, Gunasena H, Cheng Z, Espejo R, Crowe SE, Ernst PB, Reyes VE. *Helicobacter pylori* urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J Immunol* 2000; **165**: 1918-1924
6 **Wang J,** Fan X, Lindholm C, Bennett M, O'Connoll J, Shanahan F, Brooks EG, Reyes VE, Ernst PB. *Helicobacter pylori* modulates lymphoepithelial cell interactions leading to epithelial cell damage through Fas/Fas ligand interactions. *Infect Immun* 2000; **68**: 4303-4311
7 **Rudi J**, Kuck D, Strand S, von Herbay A, Mariani SM, Krammer PH, Galle PR, Stremmel W. Involvement of the CD95 (APO-1/Fas) receptor and ligand system in *Helicobacter pylori*-induced gastric epithelial apoptosis. *J Clin Invest* 1998; **102**: 1506-1514
8 **Crowe SE**, Alvarez L, Dytoc M, Hunt RH, Muller M, Sherman P, Patel J, Jin Y, Ernst PB. Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection in vitro. *Gastroenterology* 1995; **108**: 65-74
9 **Hall PA**, Coates PJ, Ansari B, Hopwood D. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* 1994; **107** (Pt 12): 3569-3577
10 **Shirin H**, Moss SF. *Helicobacter pylori* induced apoptosis. *Gut* 1998; **43**: 592-594
11 **Wong BC**, Ching CK, Lam SK, Li ZL, Chen BW, Li YN, Liu HJ, Liu JB, Wang BE, Yuan SZ, Xu CP, Hou XH, Zhang AT, Zheng ZT. Differential north to south gastric cancer-duodenal ulcer gradient in China. China Ulcer Study Group. *J Gastroenterol Hepatol* 1998; **13**: 1050-1057
12 **Malaty HM**, Kim JG, El-Zimaity HM, Graham DY. High prevalence of duodenal ulcer and gastric cancer in dyspeptic patients in Korea. *Scand J Gastroenterol* 1997; **32**: 751-754
13 **Ye W**, Held M, Lagergren J, Engstrand L, Blot WJ, McLaughlin JK, Nyrén O. *Helicobacter pylori* infection and gastric atrophy: risk of adenocarcinoma and squamous-cell carcinoma of the esophagus and adenocarcinoma of the gastric cardia. *J Natl Cancer Inst* 2004; **96**: 388-396
14 **Kerr C**. *H Pylori* protects against oesophageal cancer. *Lancet Oncol* 2003; **4**: 391
15 **Suzuki H**, Ishii H. Role of apoptosis in *Helicobacter pylori*-associated gastric mucosal injury. *J Gastroenterol Hepatol* 2000; **15** Suppl: D46-D54
16 **Ledig S**, Wagner S, Manns MP, Beil W, Athmann C. Role of the receptor-mediated apoptosis in *Helicobacter pylori* in gastric epithelial cells. *Digestion* 2004; **70**: 178-186
17 **Jones NL**, Day AS, Jennings H, Shannon PT, Galindo-Mata E, Sherman PM. Enhanced disease severity in *Helicobacter pylori*-infected mice deficient in Fas signaling. *Infect Immun* 2002; **70**: 2591-2597
18 **Xia HH**, Talley NJ. Apoptosis in gastric epithelium induced by *Helicobacter pylori* infection: implications in gastric carcinogenesis. *Am J Gastroenterol* 2001; **96**: 16-26
19 **Blancheteau V**, Charron D, Mooney N. HLA class II signals sensitize B lymphocytes to apoptosis via Fas/CD95 by increasing FADD recruitment to activated Fas and activation of caspases. *Hum Immunol* 2002; **63**: 375-383
20 **Catlett IM**, Xie P, Hostager BS, Bishop GA. Signaling through MHC class II molecules blocks CD95-induced apoptosis. *J Immunol* 2001; **166**: 6019-6024
21 **Beswick EJ**, Bland DA, Suarez G, Barrera CA, Fan X, Reyes VE. *Helicobacter pylori* binds to CD74 on gastric epithelial cells and stimulates interleukin-8 production. *Infect Immun* 2005; **73**: 2736-2743
22 **Bäckström A,** Lundberg C, Kersulyte D, Berg DE, Borén T, Arnqvist A. Metastability of *Helicobacter pylori* bab adhesin genes and dynamics in Lewis b antigen binding. *Proc Natl Acad Sci USA* 2004; **101**: 16923-16928
23 **Ilver D**, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Borén T. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* 1998; **279**: 373-377
24 **Clyne M**, Dillon P, Daly S, O'Kennedy R, May FE, Westley BR, Drumm B. *Helicobacter pylori* interacts with the human single-domain trefoil protein TFF1. *Proc Natl Acad Sci USA* 2004; **101**: 7409-7414

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BASIC RESEARCH

Effects of Ca^{2+} channel blockers on store-operated Ca^{2+} channel currents of Kupffer cells after hepatic ischemia/reperfusion injury in rats

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Abstract

AIM: To study the effects of hepatic ischemia/reperfusion (I/R) injury on store-operated calcium channel (SOC) currents (I_{SOC}) in freshly isolated rat Kupffer cells, and the effects of Ca^{2+} channel blockers, 2-aminoethoxydiphenyl borate (2-APB), SK&F96365, econazole and miconazole, on I_{SOC} in isolated rat Kupffer cells after hepatic I/R injury.

METHODS: The model of rat hepatic I/R injury was established. Whole-cell patch-clamp techniques were performed to investigate the effects of 2-APB, SK&F96365, econazole and miconazole on I_{SOC} in isolated rat Kupffer cells after hepatic I /R injury.

RESULTS: I/R injury significantly increased I_{SOC} from -80.4 ± 25.2pA to -159.5 ± 34.5pA ($^bP < 0.01$, $n = 30$). 2-APB (20, 40, 60, 80, 100 μmol/L), SK&F96365 (5, 10, 20, 40, 50 μmol/L), econazole (0.1, 0.3, 1, 3, 10 μmol/L) and miconazole (0.1, 0.3, 1, 3, 10 μmol/L) inhibited I_{SOC} in a concentration-dependent manner with IC50 of 37.41 μmol/L ($n = 8$), 5.89 μmol/L ($n = 11$), 0.21 μmol/L ($n = 13$), and 0.28 μmol/L ($n = 10$). The peak value of I_{SOC} in the I-V relationship was decreased by the blockers in different concentrations, but the reverse potential of I_{SOC} was not transformed.

CONCLUSION: SOC is the main channel for the influx of Ca^{2+} during hepatic I/R injuries. Calcium channel blockers, 2-APB, SK&F96365, econazole and miconazole, have obviously protective effects on I/R injury, probably by inhibiting Isoc in Kupffer cells and preventing the activation of Kupffer cells.

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Key words: Kupffer cell; Ischemia/reperfusion; Store-operated calcium channel currents; 2-aminoethoxydiphenyl borate; SK&F96365; Econazole; Miconazole

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INTRODUCTION

In clinical practice of hepatobiliary surgery, various factors, such as shock, inflammation, hepatic trauma, operation of liver and biliary tract (in case of necessities of interrupting hepatic portal vein), liver transplantation, are mutually related to common pathophysiological procedures, named hepatic ischemia/reperfusion (I/R) injury. Kupffer cells play an important role in hepatic I/R injury, hepatic I/R injury can be relieved when Kupffer cells are inactivated, and store-operated Ca^{2+} channels (SOC) are present both in almost all non-excitable cells and in some excitable cells[1-4], but there is no report about SOC in Kupffer cells. 2-APB, SK&F96365, econazole and miconazole have been used as the blockers of SOC in many cells. In the present study, we investigated the effects of hepatic I/R injury on store-operated calcium channel currents (Isoc) in freshly isolated rat Kupffer cells, and the effects of calcium channel blockers, 2-APB, SK&F96365, econazole and miconazole on Isoc in isolated rat Kupffer cells after hepatic I/R injury.

MATERIALS AND METHODS

Materials

Male Sprague Dawley rats (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), weighing 200-300 g, were used in all experiments.

Thapsigargin was obtained from Alexis Company. 2-aminoethoxydiphenyl borate (2-APB), SK&F96365 were obtained from Merck KcaA (Darmstadt, Germany). Collangenase IV, HEPES, EGTA, trypan blue, econazole, miconazole, CsCl, CsOH and the other chemicals were from Sigma (USA). The pipette solution contained 120.0 mmol/L CsCl, 1.0 mmol/L $MgCl_2$, 10.0 mmol/L EGTA, 10.0 mmol/L HEPES, 2 μmol/L thasigargin, pH 7.3 (adjusted with CsOH). The bath solution contained 145 mmol/L NaCl, 2.8 mmol/L KCl, 10.0 mmol/L $CaCl_2$, 1.0 mmol/L CsCl, 2.0 mmol/L $MgCl_2$, 10.0 mmol/L glucose, 10.0 mmol/L HEPES, pH 7.3 (adjusted with NaOH). Ca^{2+}-Mg^{2+}-free Krebs-ringer-HEPES buffer solution contained 5 mmol/L KCl, 1 mmol/L KH_2PO_4, 115 mmol/L NaCl, 25 mmol/L HEPES, 0.5 mmol/L EGTA, pH 7.3 (adjusted with NaOH).

Hepatic I/R injury model

The model of rat partial hepatic I/R injury was established according to the procedures reported by Colletti *et al*[5] with minor modifications. Briefly, adult animals were anesthetized with pentobarbital sodium (50 mg/kg) and heparinized (1 U/g) via an intraperitoneal injection. A midline laparotomy was performed, then an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad three lobes of liver. After 20 min for hepatic ischemia, the clip was removed followed by 40 min for hepatic reperfusion. Sham-operated control animals were treated in an identical fashion with the omission of vascular occlusion.

Cell preparation

Rat kupffer cells were enzymatically isolated from SD rats. Briefly, after the model was achieved, the portal vein and inferior vena cava were cannulated. The liver was initially perfused at a flow rate of 20-30 mL/min with a constant-flow system with modified oxygenated Ca^{2+}-Mg^{2+}-free Krebs-ringer-HEPES buffer solution (37℃) for about 5 min, followed by Krebs-ringer-HEPES buffer solution (37℃) containing collagenase IV for 10 min. After the perfusions, cephalad three lobes of the liver were excised and minced in Ca^{2+}-Mg^{2+}-free Krebs-ringer-HEPES solution (37℃) for 20 min. The cells were filtered through a 200 μm nylon mesh, and washed by centrifugation at 700 g for 7 min. Then we reserved sediment and poured about 20-30 mL PBS into the sediment, washed it by centrifugation at 50 g for 4 min, reserved supernatant and washed it by centrifugation at 700 g for 5 min, plated the sediment on glass cover-slips and incubated it at 37℃ in DMEM for 1-2 h. Then the cells were washed 3-5 times with the bath solution. The cells attached to the bottom of glass cover-slips were kupffer cells. The spherical, smooth cells were used for the whole-cell patch-clamp studies. All experiments were performed at room temperature (20-24℃).

Electrophysiological recordings

An automatic micropipette puller (Model P-97, Sutter Instruments, Novato, CA) was used to pull the electrodes. The resistance of the capillary glass electrodes (GG217, Would Precision, USA) used was 4-8 MΩ when filled with internal solution. An Axopatch 200B amplifier (Axon Instruments, USA) was used to record whole-cell currents with the filter set at 3 kHz, digitized at 5 kHz. The protocol of a series of depolarizing pulses from a holding potential of 0 mV to different membrane potentials (-120 mV to +80 mV) with a 20 mV increment was used for voltage clamp. Data analysis was performed using software (pCLAMP9.0, Axon Instruments, USA). The data were stored in computer for subsequent analysis. The blockers were added to the place around the cells with a rapid solution changer to investigate their effects on Isoc.

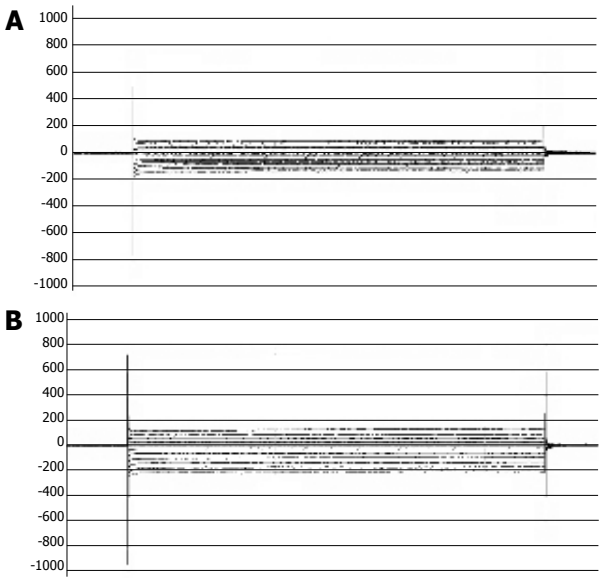

Figure 1 Whole-cell currents measured at the test potential from -120 mV to + 80 mV in sham-operated control (**A**) and in rats with ischemia/reperfusion injuries (**B**).

Statistical analysis

All values were expressed as mean ± SD. Appropriate *t*-test was used for the statistical analysis. $^aP < 0.05$ and $^bP < 0.01$ were considered statistically significant and apparently significant respectively.

RESULTS

Viability of isolated rat Kupffer cells

The isolated Kupffer cells showed polymorphism with typical polygon-like and star-like shapes. The purity and adhesion rate were 85% and 39.4%, respectively. The viability was over 80%. The isolated rat Kupffer cells were suitable for whole-cell patch clamp technique.

Isoc in isolated rat Kupffer cells

After the whole-cell configuration was established, cell membrane potential was clamped at 0 mV, and different test potentials from -120 to +80 mV with a 20 mV increment at a frequency of 0.2Hz were applied. Following break-in, the currents in ischemia/reperfusion group (-159.5 ± 34.5 pA, n = 30) were greater than those in sham-operated control group (-80.4 ± 25.2 pA, n = 30) at the test potential of -100 mV ($^bP < 0.01$, Figure 1).

Table 1 Effects of 2-APB on Isoc at the test potential of -100 mV (mean ± SD, *n* = 8)

Group (μmol/L)	Peak current (pA)	*t*	Inhibitive rate (%)
Control	-227.8 ± 68.5		
2-APB (20)	-139.6 ± 52.9[b]	7.6	39.0 ± 10.3
2-APB (40)	-109.6 ± 36.4[b]	6.2	50.8 ± 12.2
2-APB (60)	-88.1 ± 37.6[b]	6.7	61.0 ± 15.1
2-APB (80)	-72.4 ± 37.1[b]	6.8	67.7 ± 15.1
2-APB (100)	-57.8 ± 33.4[b]	6.9	73.7 ± 14.3

[b]$P < 0.01$ *vs* control.

Table 2 Effects of SK&F96365 on Isoc at the test potential of -100 mV (mean ± SD, *n* = 11)

Group (μmol/L)	Peak current (pA)	*t*	Inhibitive rate (%)
Control	-161.7 ± 58.7		
SK&F96365 (5)	-82.6 ± 29.5[b]	5.3	46.1 ± 17.3
SK&F96365 (10)	-57.0 ± 24.9[b]	6.1	61.2 ± 20.5
SK&F96365 (20)	-47.1 ± 20.5[b]	6.5	67.2 ± 19.5
SK&F96365 (40)	-35.6 ± 15.4[b]	7.1	74.8 ± 15.2
SK&F96365 (50)	-25.3 ± 11.9[b]	7.7	82.1 ± 11.0

[b]$P < 0.01$ *vs* control.

Table 3 Effects of econazole on Isoc at the test potential of -100 mV (mean ± SD, *n* = 13)

Group (μmol/L)	Peak current (pA)	*t*	Inhibitive rate (%)
Control	-147.2 ± 35.7		
econazole (0.1)	-83.5 ± 22.8[b]	6.7	42.0 ± 15.1
econazole (0.3)	-64.7 ± 18.9[b]	8.7	54.6 ± 14.7
econazole (1)	-56.6 ± 19.9[b]	9.8	60.7 ± 13.7
econazole (3)	-47.9 ± 18.2[b]	10.3	66.5 ± 13.5
econazole (10)	-39.9 ± 15.9[b]	11.1	71.9 ± 12.6

[b]$P < 0.01$ *vs* control.

Table 4 Effects of miconazole on Isoc at the test potential of -100 mV (mean ± SD, *n* = 10)

Group (μmol/L)	Peak current (pA)	*t*	Inhibitive rate (%)
Control	-133.2 ± 32.0		
miconazole (0.1)	-86.4 ± 31.7[b]	7.1	35.6 ± 14.9
miconazole (0.3)	-63.8 ± 23.4[b]	10.7	52.3 ± 10.0
miconazole (1)	-45.9 ± 13.1[b]	9.2	64.5 ± 10.6
miconazole (3)	-36.8 ± 11.4[b]	9.9	71.3 ± 10.2
miconazole (10)	-29.7 ± 10.5[b]	9.9	76.5 ± 9.4

[b]$P < 0.01$ *vs* control.

But the I-V curve relation and reversal potential were not transformed (Figure 2).

Effects of 2-APB on Isoc

In order to observe the effects of 2-APB on Isoc, 2-APB with different concentrations (20, 40, 60, 80, 100 μmol/L) was respectively added to periphery of Kupffer cells after the whole cell configuration was established. According to the whole-cell currents at the test potential of -100mV (Table 1), Isoc was blocked by 2-APB in a concentration-dependent fashion with the IC50 of 37.41 μmol/L. But the I-V curve relation and reversal potential were not transformed (Figure 3A).

Effects of SK&F96365 on Isoc

In order to observe the effects of SK&F96365 on Isoc, SK&F96365 with different concentrations (5, 10, 20, 40, 50 μmol/L) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100mV (Table 2), Isoc was blocked by SK&F96365 in a concentration-dependent fashion with the IC50 of 5.89 μmol/L. But the I-V curve relation and reversal potential were not transformed (Figure 3B).

Effects of econazole on Isoc

In order to observe the effects of econazole on Isoc, econazole with different concentrations (0.1, 0.3, 1, 3, 10 μmol/L) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100 mV (Table 3), Isoc was blocked by econazole in a concentration-dependent fashion with the IC50 of 0.21 μmol/L. But the I-V curve relation and reversal potential were not transformed (Figure 3C).

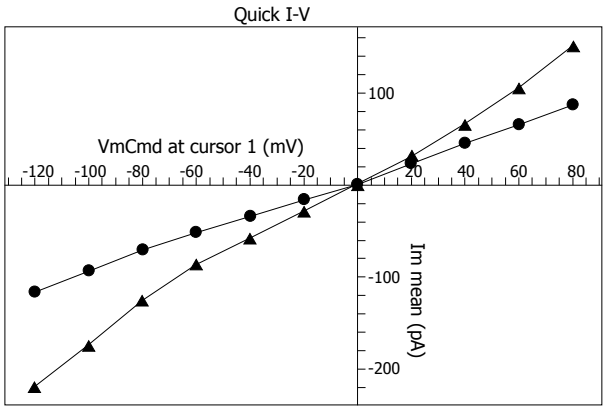

Figure 2 I-V curve of whole-cell currents measured at -100 mV test potential. [b]$P < 0.01$ *vs* sham-operated control (●) (▲I/R).

Effects of miconazole on Isoc

In order to observe the effects of miconazole on Isoc, miconazole with different concentrations (0.1, 0.3, 1, 3, 10 μmol/L) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100 mV (Table 4), Isoc was blocked by miconazole in a concentration-dependent fashion with the IC50 of 0.28 μmol/L. But the I-V curve relation and reversal potential were not transformed (Figure 3D).

DISCUSSION

In this study, a rat hepatic I/R injury model was established. Kupffer cells were isolated and Isoc in Kupffer cells were detected.

Isoc can be activated by two ways, one is active way

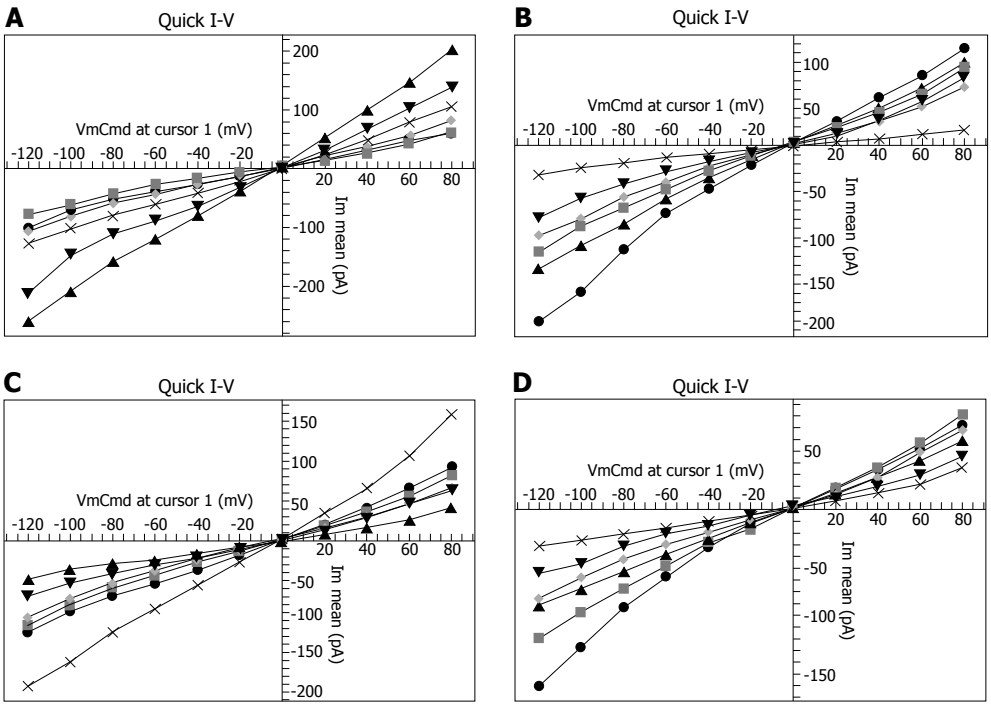

Figure 3 I-V curve of whole-cell currents at -100 mV test potential affected by 2-APB (**A**) (▲I/R, ▼20 μmol/L, × 40 μmol/L, ◆60 μmol/L, ●80 μmol/L, ■100 μmol/L), SK&F96365 (**B**) (●I/R, ▲5 μmol/L, ■10 μmol/L, ◆20 μmol/L, ▼40 μmol/L, × 50 μmol/L), econazole (**C**) and (× I/R, ●0.1 μmol/L, ■0.3 μmol/L, ◆1 μmol/L, ▼3 μmol/L, ▲10 μmol/L), miconazole (**D**) in a concentration-dependent inhibiting fashion (●I/R, ■0.1 μmol/L, ▲0.3 μmol/L, ◆1 μmol/L, ▼3 μmol/L, × 10 μmol/L).

induced by IP_3[6-8], and the other way is EGTA induced by thasigargin[9]. The latter was chosen in our study.

Hepatic I/R injury can cause calcium overload in liver cells[10-12]. Kupffer cells are considered to play a major role in hepatic I/R injury. The hepatic partial I/R process leads to activation of kupffer cells in ischemic and nonischemic areas of the liver, superoxide generation and proinflammatory cytokine production in Kupffer cells[13]. An excess of reactive oxygen species (ROS) is generated by Kupffer cells activated during hepatic I/R injuries. All these events cause pathophysiological changes initiating a cascade of hepatocellular injury, necrosis, apoptosis, and subsequent inflammation[14-16]. Inhibiting activation of Kupffer cells can relieve hepatic I/R injuries. Calcium overload is one of the important reasons for activation of Kupffer cells. In our study, I/R injuries could significantly increase Isoc of the Kupffer cells, suggesting that SOC has a close relation with hepatic I/R injuries and is the main channel for the influx of Ca^{2+} during calcium overload. *Broad et al*[17] showed that phospolipase C and polyphosphoinositides can activate capacitative calcium entry (CCE). Recent findings indicate that receptor-mediated activation of phospholipase C in intact cells activates TRPC3 diacylglycerol production, independently of G proteins, protein kinase C, or inositol 1, 4, 5-trisphosphate[18]. The last source of ion channel is the transient receptor potential (TPR) channel family, which forms non-selective cations[19] all these indicate that hepatic I/R injuries activate phospholipase C which induces activation of store-operated calcium channels or TRPC3 channels, finally leading to calcium overload of Kupffer cells which could exacerbate hepatic I/R injuries.

Sequentially, Isoc of Kupffer cells after hepatic I/R injuries could be blocked by 2-APB, SK&F96365, econazole and miconazole in a concentration-dependent fashion.

2-APB has the restraint effect on Isoc in many kinds of cells[20,21] and is a blocker of SOC and TRP channels[22-24]. Our data have shown that Isoc of Kupffer cells after hepatic I/R injuries is blocked by 2-APB in a concentration-dependent fashion from 20 μmol/L to 100 μmol/L. 2-APB inhibits SOC by IP_3 way[25,26]. Recent studies indicate that 2-APB may act as a direct blocker rather than as an IP_3 receptor antagonist while Icrac activity is rapidly blocked by extracellular 2-APB, but not by intracellular 2-APB[27-30]. Furthermore, *Broad et al*[17] showed 2-APB abolishes CCE induced by thapsigargin even in DT40 cells deficient for all isoforms of IP_3 receptor, consistent with a direct action of 2-APB on the SOC themselves. Our data also suggest that 2-APB may act as a direct blocker for SOC. Therefore, 2-APB protects Kupffer cells against being activated after hepatic I/R injuries by blocking Isoc.

SK&F96365 could block Isoc in many cells such as HL-60 cells, thyroid gland FRTL-5 cells, thrombocytes as well as voltage-dependent Ca^{2+} channels (VDCCs) in GH3 pituitary cells and smooth muscle cells[31], suggesting that SK&F96365 can be used as a blocker of SOC in non-excited cells without VDCCs. VDCCs have been proved to be Kupffer cells, but Ca^{2+} current induced by EGTA and thapsigargin could not be blocked by verapamil (a kind of blocker of VDCCs). In our study, Isoc of Kupffer cells after hepatic I/R injuries was blocked by SK&F96365 in a concentration-dependent fashion. The mechanism is not completely clear. Since SK&F96365 could block two different channels, SOC and VDCCs, it may act on the SOC directly.

Econazole and miconazole decrease intracellular calcium levels after activation of SOC channels[32] and block Isoc in Jurkat T-cells, HL-60 leukocytes, HEL cells, *etc*. Our data demonstrate that Isoc of Kupffer cells after hepatic I/R injuries is blocked by econazole and miconazole in a

concentration-dependent fashion.

In conclusion, hepatic I/R injuries can activate Kupffer cells, probably by increasing Isoc in Kupffer cells and activated Kupffer cells exacerbate hepatic I/R injuries. 2-APB, SK&F96365, econazole and miconazole can inhibit Isoc of Kupffer cells after hepatic I/R injuries in a concentration-dependent fashion. They have obvious protective effects on I/R injury, probably by inhibiting Isoc of Kupffer cells and preventing activation of Kupffer cells.

REFERENCES

1 **Vazquez G**, Wedel BJ, Aziz O, Trebak M, Putney JW Jr. The mammalian TRPC cation channels. *Biochim Biophys Acta* 2004; **1742**: 21-36

2 **Penner R**, Fleig A. Store-operated calcium entry: a tough nut to CRAC. *Sci STKE* 2004; **2004:** pe38

3 **Putney JW Jr**. Store-operated calcium channels: how do we measure them, and why do we care? *Sci STKE* 2004; **2004:** pe37

4 **Gregory RB**, Hughes R, Riley AM, Potter BV, Wilcox RA, Barritt GJ. Inositol trisphosphate analogues selective for types I and II inositol trisphosphate receptors exert differential effects on vasopressin-stimulated Ca2+ inflow and Ca2+ release from intracellular stores in rat hepatocytes. *Biochem J* 2004; **381**: 519-526

5 **Colletti LM**, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr. Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. *J Clin Invest* 1990; **85**: 1936-1943

6 **Gregory RB**, Sykiotis D, Barritt GJ. Evidence that store-operated Ca2+ channels are more effective than intracellular messenger-activated non-selective cation channels in refilling rat hepatocyte intracellular Ca2+ stores. *Cell Calcium* 2003; **34**: 241-251

7 **Gregory RB**, Barritt GJ. Evidence that Ca2+-release-activated Ca2+ channels in rat hepatocytes are required for the maintenance of hormone-induced Ca2+ oscillations. *Biochem J* 2003; **370**: 695-702

8 **Wissing F**, Nerou EP, Taylor CW. A novel Ca2+-induced Ca2+ release mechanism mediated by neither inositol trisphosphate nor ryanodine receptors. *Biochem J* 2002; **361**: 605-611

9 **Rychkov G**, Brereton HM, Harland ML, Barritt GJ. Plasma membrane Ca2+ release-activated Ca2+ channels with a high selectivity for Ca2+ identified by patch-clamp recording in rat liver cells. *Hepatology* 2001; **33**: 938-947

10 **Silomon M**, Pizanis A, Rose S. Oxyradical-mediated hepatocellular Ca2+ alterations during hemorrhagic shock and resuscitation. *Shock* 1999; **11**: 193-198

11 **Sung YJ**, Sung Z, Ho CL, Lin MT, Wang JS, Yang SC, Chen YJ, Lin CH. Intercellular calcium waves mediate preferential cell growth toward the wound edge in polarized hepatic cells. *Exp Cell Res* 2003; **287**: 209-218

12 **Ben Abdennebi H**, Steghens JP, Hadj-Aïssa A, Barbieux A, Ramella-Virieux S, Gharib C, Boillot O. A preservation solution with polyethylene glycol and calcium: a possible multiorgan liquid. *Transpl Int* 2002; **15**: 348-354

13 **Nakamitsu A**, Hiyama E, Imamura Y, Matsuura Y, Yokoyama T. Kupffer cell function in ischemic and nonischemic livers after hepatic partial ischemia/reperfusion. *Surg Today* 2001; **31**: 140-148

14 **Jambrina E**, Alonso R, Alcalde M, del Carmen Rodríguez M, Serrano A, Martínez-A C, García-Sancho J, Izquierdo M. Calcium influx through receptor-operated channel induces mitochondria-triggered paraptotic cell death. *J Biol Chem* 2003; **278**: 14134-14145

15 **Lemasters JJ**, Qian T, He L, Kim JS, Elmore SP, Cascio WE, Brenner DA. Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. *Antioxid Redox Signal* 2002; **4**: 769-781

16 **Nieminen AL**. Apoptosis and necrosis in health and disease: role of mitochondria. *Int Rev Cytol* 2003; **224**: 29-55

17 **Broad LM**, Braun FJ, Lievremont JP, Bird GS, Kurosaki T, Putney JW Jr. Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. *J Biol Chem* 2001; **276**: 15945-15952

18 **Trebak M**, St J Bird G, McKay RR, Birnbaumer L, Putney JW Jr. Signaling mechanism for receptor-activated canonical transient receptor potential 3 (TRPC3) channels. *J Biol Chem* 2003; **278**: 16244-16252

19 Clapham DE, Runnels LW, Strübing C. The TRP ion channel family. *Nat Rev Neurosci* 2001; **2**: 387-396

20 **Wilson SM**, Mason HS, Ng LC, Montague S, Johnston L, Nicholson N, Mansfield S, Hume JR. Role of basal extracellular Ca2+ entry during 5-HT-induced vasoconstriction of canine pulmonary arteries. *Br J Pharmacol* 2005; **144**: 252-264

21 **Tamareille S**, Mignen O, Capiod T, Rücker-Martin C, Feuvray D. High glucose-induced apoptosis through store-operated calcium entry and calcineurin in human umbilical vein endothelial cells. *Cell Calcium* 2006; **39**: 47-55

22 **Iwasaki H**, Mori Y, Hara Y, Uchida K, Zhou H, Mikoshiba K. 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels* 2001; **7**: 429-439

23 **Bootman MD**, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM. 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. *FASEB J* 2002; **16**: 1145-1150

24 **Braun FJ**, Aziz O, Putney JW Jr. 2-aminoethoxydiphenyl borane activates a novel calcium-permeable cation channel. *Mol Pharmacol* 2003; **63**: 1304-1311

25 **Maruyama T**, Kanaji T, Nakade S, Kanno T, Mikoshiba K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. *J Biochem* 1997; **122**: 498-505

26 **Wang JP**, Tseng CS, Sun SP, Chen YS, Tsai CR, Hsu MF. Capsaicin stimulates the non-store-operated Ca2+ entry but inhibits the store-operated Ca2+ entry in neutrophils. *Toxicol Appl Pharmacol* 2005; **209**: 134-144

27 **Ma HT**, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K, Gill DL. Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. *Science* 2000; **287**: 1647-1651

28 **Braun FJ**, Broad LM, Armstrong DL, Putney JW Jr. Stable activation of single Ca2+ release-activated Ca2+ channels in divalent cation-free solutions. *J Biol Chem* 2001; **276**: 1063-1070

29 **Kukkonen JP**, Lund PE, Akerman KE. 2-aminoethoxydiphenyl borate reveals heterogeneity in receptor-activated Ca(2+) discharge and store-operated Ca(2+) influx. *Cell Calcium* 2001; **30**: 117-129

30 **Lievremont JP**, Bird GS, Putney JW Jr. Mechanism of inhibition of TRPC cation channels by 2-aminoethoxydiphenyl borane. *Mol Pharmacol* 2005; **68**: 758-762

31 **Merritt JE**, Armstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa-Chamiec A, Leigh BK, McCarthy SA, Moores KE, Rink TJ. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J* 1990; **271**: 515-522

32 **Harper JL**, Shin Y, Daly JW. Loperamide: a positive modulator for store-operated calcium channels? *Proc Natl Acad Sci USA* 1997; **94**: 14912-14917

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CLINICAL RESEARCH

Colorectal mucosal histamine release by mucosa oxygenation in comparison with other established clinical tests in patients with gastrointestinally mediated allergy

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Abstract

AIM: This study evaluated colorectal mucosal histamine release in response to blinded food challenge-positive and -negative food antigens as a new diagnostic procedure.

METHODS: 19 patients suffering from gastrointestinally mediated allergy confirmed by blinded oral provocation were investigated on grounds of their case history, skin prick tests, serum IgE detection and colorectal mucosal histamine release by *ex vivo* mucosa oxygenation. Intact tissue particles were incubated/stimulated in an oxygenated culture with different food antigens for 30 min. Specimens challenged with anti-human immunoglobulin E and without any stimulus served as positive and negative controls, respectively. Mucosal histamine release (% of total biopsy histamine content) was considered successful (positive), when the rate of histamine release from biopsies in response to antigens reached more than twice that of the spontaneous release. Histamine measurement was performed by radioimmunoassay.

RESULTS: The median (range) of spontaneous histamine release from colorectal mucosa was found to be 3.2 (0.1%-25.8%) of the total biopsy histamine content. Food antigens tolerated by oral provocation did not elicit mast cell degranulation 3.4 (0.4%-20.7%, P = 0.4), while anti-IgE and causative food allergens induced a significant histamine release of 5.4 (1.1%-25.6%, P = 0.04) and 8.1 (1.5%-57.9%, P = 0.008), respectively. 12 of 19 patients (63.1%) showed positive colorectal mucosal histamine release in accordance with the blinded oral challenge responding to the same antigen (s), while the specificity of the functional histamine release to accurately recognise tolerated foodstuffs was found to be 78.6%. In comparison with the outcome of blinded food challenge tests, sensitivity and specificity of history (30.8% and 57.1%), skin tests (47.4% and 78.6%) or antigen-specific serum IgE determinations (57.9% and 50%) were found to be of lower diagnostic accuracy in gastrointestinally mediated allergy.

CONCLUSION: Functional testing of the reactivity of colorectal mucosa upon antigenic stimulation in patients with gastrointestinally mediated allergy is of higher diagnostic efficacy.

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Key words: Gut; Histamine release; Mucosa oxygenation; Food allergy diagnostics; Gastrointestinally mediated allergy

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INTRODUCTION

Gastrointestinal complaints after the ingestion of certain foodstuffs often pose diagnostic problems in various clinical situations such as food hypersensitivity, enzyme deficiencies, irritable bowel syndrome, inflammatory bowel disease, dyspepsia, eosinophilic gastroenteritis and several others. However, identification of immunologically mediated food hypersensitivity at the gastrointestinal level remains problematic, since skin tests and allergen specific serum IgE detection (e.g. RAST) may fail to show clear signs of food-specific sensitisation[1-5] and do not necessarily indicate symptomatic food allergy. This is also valid for functional tests using blood cells (basophil histamine, or leucotriene release), lymphocyte transformation tests or measurement of mediators in blood or serum[6-8]. Oral provocation, referred to as the 'gold standard' for food allergy diagnosis, is both time consuming and cost intensive, may put the patient at a

more or less severe risk, allows only one food to be tested per day, is often difficult to evaluate and is without doubt an unpopular and irritating procedure for the patient[1,5,8].

Consequently, different methodical approaches have emerged for the improvement and acceleration of the cumbersome diagnostic way to identify patients with food allergy primarily involving the gastrointestinal tract. Already in 1942, 1984 and 1997, direct endoscopic observation has repeatedly been claimed to be of diagnostic value for recognition of food allergy when antigenic solutions were applied to the intestinal mucosa[9-11]. However, direct endoscopic evaluation of allergen application and endoscopic or fluoroscopic balloon perfusion techniques harbour similar disadvantages to those experienced in oral provocation tests. All of these tests require special conditions with strict medical supervision because of the risk of allergic reactions *in vivo* during endoscopy or allergen perfusion[10-12]. Only a few allergens can be tested during one examination and, in case of an endoscopic allergen injection, the endoscopic procedure is prolonged by at least 20 min, providing a higher risk of procedure-related complications, bacterial translocation and discomfort to the patient[10-14]. Finally, the outcome of these endoscopic methods and the results obtained from double-blind, placebo-controlled food challenge procedures have never been directly compared on a scientific basis. Nevertheless, this is absolutely necessary for an appropriate evaluation in routine practice with respect to their diagnostic effectiveness[1,5,8].

Another possibility to identify food allergy at the gastrointestinal tract was described in 1989. Baenkler *et al* used endoscopically taken samples from the duodenum to show antigen-induced histamine release *ex vivo*[15,16]. Since many patients with suspected gastrointestinally mediated allergy (GMA) have to undergo endoscopic procedures for differential diagnostic reasons, this approach has the advantage that the principle demonstration of a food-induced mediator release can be performed outside of the patient, thus avoiding the risk of allergic reactions *in vivo*. In addition, several food antigens can be tested simultaneously without further burden to the patient[15-17]. However, performance of functional histamine release experiments requires certain laboratory equipment and, initially, should be compared with the 'gold standard' for food allergy diagnostics before introducing this approach into clinical practice.

Similar to Baenkler's approach using duodenal mucosa, functional histamine release should also be expected from mucosa of other regions of the intestine[15-17], provided that these tissue samples contain large enough numbers of (mucosal) mast cells. However, *ex vivo* histamine release from viable tissue samples of the lower gastrointestinal tract in response to nutritive antigens has not yet been studied together with the standardised *in vivo* oral challenge tests in order to provide a direct comparison of the two diagnostic methods. For this reason, this study investigated the rate of histamine release from colorectal mucosal samples in a group of patients with proven food allergy and compared the results of *ex vivo* mucosa oxygenation with the outcome of standardised blinded oral provocation tests.

Table 1 Patient data, allergens used for oral provocation, colorectal mucosal histamine release, skin prick test, serum IgE detection (RAST test), and patient's history

Pat. No.	Sex	Age	Allergen	Oral provocation	Colorectal HR	Skin test	RAST IgE	H
1WT	F	44	Cheese	+	+	-	+	?
			Rye bran	-	-	+	+	?
2DF	F	49	House dust	+	+	+	+	?
			Rye pollen	-	+	-	+	?
3OL	M	41	Soya flour	+	+	+	+	+
			Rye flour	-	-	-	+	?
4CG	M	23	Rye flour	+	+	-	-	-
5NA	F	42	Wheat flour	+	-	+	+	?
			Soya flour	-	-	-	+	?
6IL	F	43	Wheat bran	+	+	-	+	?
			Barley flour	-	-	+	-	?
7GG	F	19	Soya flour	+	+	+	+	?
			Wheat flour	-	+	-	+	?
8FJ	F	25	Soya lecithin	+	-	+	+	+
			Milk	-	-	-	+	+
9MR	F		Milk	+	-	-	-	-
			Potato	-	-	-	-	-
10MB	F	23	Wheat/rye	+	-	+	+	-
			Egg	-	-	-	-	+
11FB	F	51	Wheat flour	+	+	-	+	+
12RA	M	38	Spices	+	+	-	-	+
			Milk	-	-	-	-	+
13KF	M	32	Apple	+	+	+	+	-
			Potato	-	-	-	-	-
14AC	F	40	Nuts	+	+	-	-	-
			Maize	-	-	-	+	-
15KF	M	38	Nuts	+	-	+	+	-
16TM	F	28	Nuts	+	-	-	-	-
17SJ	F	37	Nuts	+	-	-	-	-
18LS	M	44	Nuts	+	+	-	-	-
			Rice	-	-	-	-	-
19SL	M	29	Wheat flour	+	+	+	-	?
			Potato	-	+	+	-	?

HR: histamine release; no.: number; pat.: patient; H: patients' history with regard to causative allergens and tolerated foodstuff.

MATERIALS AND METHODS

Patients

A total of 19 patients (7 male, 12 female; median age 38.0, range 19-51 years) were included in this study (Tables 1 and 2). All patients gave their informed consent and the study protocol was approved by the local ethics committee (No. 331). All patients (100%) reported abdominal symptoms, nausea, pain, vomiting and/or diarrhoea (98%) after certain meals, while postprandial extraintestinal signs of allergy such as skin reactions, asthma bronchiale and allergic rhinoconjunctivitis occurred only in a small percentage of patients (32%). Every patient was assessed on grounds of their history and detailed skin prick tests of environmental allergens (moulds, fibres, bacteria, pollen, dust) and food allergens (fish, fruit, vegetables, grains and different types of wholemeal, flour, bran, tea, coffee, eggs, milk, and cheese). Serum IgE detection of the putative allergens was performed according to the patients' history or skin tests (Tables 1 and 2). In the case of uncertainties about non-tolerated foods, tests were conducted for basic foodstuff. Case history, skin test reactions and RAST results were then compared with the outcome of oral

Table 2 Clinical symptoms induced by blinded, placebo-controlled food challenge, atopy status and predominant type of allergy according to Coombs and Gell

Pat. No.	Main symptoms	Atopy status	Type of allergy
1	Diarrhoea, flush, pruritus	-	Type I (systemic IgE)
2	Abdominal pain, loose stools bloating	+	Type I (systemic IgE)
3	Vomiting, diarrhoea	+	Type I (systemic IgE)
4	Abdominal pain, urticaria	-	Type I (local IgE)
5	Diarrhoea, abdominal pain dyspepsia, vomiting	+	Type I (systemic IgE)
6	Diarrhoea	-	Type I (systemic IgE)
7	Vomiting, loose stools right lower quadrant pain	+	Type I (systemic IgE)
8	Profuse watery diarrhoea	-	Type I (systemic IgE)
9	Diarrhoea, bloating, tachy-cardia	+	Type III (immune com-plexes present) or IV (?)
10	Pruritus, Rhinitis, tachycardia bloating, diarrhoea	-	Type I (systemic IgE)
11	Colitis, diarrhoea, arthragia rhinitis	-	Type I (systemic IgE)
12	Bloody diarrhoea, hypotension, abdominal pain, bloating	-	Type I (local IgE) and /or type III (immune complexes present)
13	Fever, diarrhoea, hypotension	+	Type I (systemic IgE)
14	Diarrhoea, vomiting, abdominal pain	-	Type IV (cellular hyper-sensitivity ?)
15	Bloating, diarrhoea, eosinophilia	+	Type I (systemic IgE)
16	Atopic eczema, diarrhoea, colitis, abdominal pain	+	Type I (local IgE) and /or type IV (cellular hyper-sensitivity ?)
17	Rhinitis, vomiting, diarrhoea	+	Type I (local IgE)
18	Diarrhoea, bloating	+	Type I (local IgE)
19	Eosinophilia, bloating, diarrhoea	+	Type I (systemic IgE)

Atopy status was defined as positive, when history or clinical manifestation of the patient gave evidence for milk crust, atopic eczema, asthma bronchiale and/or allergic rhino-conjunctivitis. For definition of the allergy type, the most dominant immunological signs were chosen to classify the ongoing allergic mechanisms in this population of patients with manifest gastrointestinally mediated allergy. However, some patients displayed symptoms that suggested more than one definitive type of allergy (see for example patient No. 9, 12, 16): Type I allergy (systemic IgE) was recognised when positive skin or antigen specific IgE levels were present in serum, type I allergy (local IgE) was diagnosed when intestinal lavage fluid contained elevated levels of IgE[20]. Type Ⅲ allergy was found in patients no. 9 and 12 who showed formation of either IgA, IgM and/or IgE immune complexes during or after allergen application by blinded food challenge. Additionally, type IV allergy was suspected in patients with heightened serum TNF levels during food challenge, and additionally in patient no. 14 who had a positive antigen-specific lymphocyte proliferation test.

challenge tests.

Food allergy was confirmed in each patient by blinded, placebo-controlled food challenge tests (BPCFC) adding the putative allergen to a basic diet containing rice, potato, oligopeptides (Survimed OPD, Fresenius, Germany) and tea. For provocation of flours (wheat, rye, soya, barley), commercially available allergen solutions for skin tests were used (Maser, Bochum, Germany). These were applied orally, while all other allergens were freshly prepared and given to the patients via a nasogastric tube[5,18].

BPCFC was performed in a standardised fashion, while patients were hospitalised. Food antigen was administered in three different doses. Initially, a 1/20 dilution of the native allergen solution was administered, followed by 1/10 of the dose 3 h later and finally, a dose of 5 mL of the full strength native allergen solution was provided[18,19]. One single food antigen was tested per day. Placebo consisted of an oligopeptide-diet (protein source: hydrolysed soybean, Survimed OPD, Germany), which was also used for base-line nutrition (minimum: 1800 kcal/d), in conjunction with a rice-potato diet in order to prevent a catabolic state[18,19]. A single blind challenge was performed in 42% (patients unaware of provocation protocol), while a double-blind challenge was carried out in 58% (patients and physicians unaware of the provocation protocol)[18,19]. Blinding of the food antigens was managed by nutritionists, who were responsible for the preparation and addition of the allergens to usually tolerated foodstuff or to the oligopeptide solution, respectively.

Physicians selected the type of food to be tested either on the basis of the patients' history, previous results of skin prick tests and RAST tests or from a list of basic foodstuff. During the provocation procedure, the patients were provided with a peripheral venous line, and all medical staff involved was trained for medical intervention in case of an anaphylactic reaction. For the definition of food allergic reactions, a modified scoring system for symptoms was applied[18] and main symptoms of patients evoked by the food challenge are listed in Table 2.

Food hypersensitivity was diagnosed only when food-specific immune events were detected through positive skin tests (mean wheal diameter of 3mm or greater than negative control[1,9,18,19]), serum RAST-IgE (⩾ class Ⅱ[8,19]) or through proof of intestinal IgE by endoscopically guided segmental lavage[18,19] in conjunction with a reproducible clinical adverse reaction to the food antigen(s) applied[1,5,8,18,19]. During BPCFC, at least one reproduction of an allergen induced clinical reaction and one or two placebo challenges were included for every patient. Whenever possible, both antigens causing clinical symptoms as well as tolerated antigens were applied to the patient, or else investigated on grounds of case history, skin tests, RAST and mucosal histamine release. In this way, a provocation allergen and a control allergen (Table 1) was determined for most patients (14 of 19 patients 74%), which enabled the direct comparison of the mucosal histamine release results with those of the BPCFC.

Before the execution of food challenge tests, additional examinations including endoscopy and histology of the upper and lower gastrointestinal tract were conducted[5,18-20]. Patients with macroscopic alterations of the mucosa or with histological signs of acute inflammation (Crohn's Disease, ulcerative colitis *etc*) were excluded from the study as well as those suffering from other digestive diseases (e.g. celiac disease, autoimmunopathy, mastocytosis, eosinophilic gastroenteritis *etc*).

At least two weeks in advance of colonoscopy and BPCFC, any antiallergenic, immunosuppressive or steroid treatments had been discontinued for all patients. Patients were prepared for colonoscopy using a commercial polyethylenglycol solution. To facilitate colonoscopy, benzodiazepins (midazolam, diazepam) and meperidine were used at a dose of 2.5-10 mg (midazolam, diazepam) and 25-150 mg (meperidine), respectively[20,21].

Colorectal mucosal histamine release

For colorectal mucosal histamine release by mucosa

oxygenation, 138 samples from the left-sided colon were obtained from all 19 patients. In 14 of 19 patients (74%), 10-12 mucosal samples were taken during colonoscopic examination. Whenever possible, 8 biopsies (4 repeats) were used for mucosa oxygenation and 2-4 for histological examination. The biopsies were immediately placed into a portable mucosa oxygenator (Intestino-Diagnostics, Erlangen, Germany) containing tubes filled with 2000 μL of oxygenated Hank's solution (pO_2 85-95 mmHg, pH 7.0, 37℃)[16,17,20]. Each incubation medium was bubbled with a steady flow of room air to ensure sufficient oxygen pressure inside the biopsy, to facilitate allergen distribution into the tissue or mediator release from the tissue and to avoid ischemic damage of the tissue[16,17,20,21].

Histamine release into the culture medium was measured at 0, 7.5, 15, and 30 min by removal of 200 μL of the supernatant at each sampling time[17,20,21]. To obtain as accurate histamine measurements as possible, each 200 μL sample was immediately denatured by heating to 95℃ for 5 min in order to destroy all histamine metabolising enzymes that may have been contained within the drawn supernatant[16,17,20,21].

Allergen induced histamine release was achieved by addition of 200 μL Hank's solution containing 5 μL of native allergen solution to the culture medium at the sampling time of 0 min, thus providing a final concentration of 0.01 μg allergen/mL. The same procedure was applied for positive control using anti-human immunoglobulin E except that 20 μL of pure anti-IgE solution (Behringwerke, Marburg, Germany) were used for dilution. The final concentration of anti-human-IgE was 0.01 μg/mL, which has previously been found to be the optimal stimulation concentration[16,17]. For negative control (spontaneous mucosal histamine release), only 200 μL Hank's solution were added to the culture medium. The stimulation procedure of the 8 samples of each patient was arranged as follows: two samples were studied for spontaneous mucosal histamine release (negative control), two for anti-IgE induced histamine release (positive control), two for a BPCFC-positive allergen (provocation allergen) and two for provocation negative, i.e. tolerated antigens (control allergen).

After a stimulation period of 30 min, the rest of the volume of 1400 μL containing the biopsy (1200 μL Hank's + 200 μL stimulus) was also heated to 95℃ for five minutes in order to determine the remaining tissue histamine content and to denature tissue histamine catabolising enzymes[17,20,21].

Histamine measurement

Histamine was measured using a sensitive and specific radioimmunoassay (Histamine RIA, Beckman-Coulter, Krefeld, Germany)[17,21,22]. The actual rate of histamine release was expressed as the percentage of the total tissue histamine content of the biopsy. This was calculated from the discharged histamine into the supernatant and the remaining histamine content in the tissue at the sampling time of 30 min[17,20-22]. Intra-assay and inter-assay coefficients of variation for the histamine radioimmunoassay (n > 150 samples) were 6.2% and 8.8% for supernatants, and 13.7% and 18.2% for detection of the remaining tissue histamine content, respectively. The individual rates of histamine release were found to vary by up to 24.0% within the same person.

Histamine content was also measured in native allergen solutions to exclude histamine contamination.

Table 3 Results from colorectal mucosal histamine release (% of total tissue histamine content) following *ex vivo* mucosa oxygenation in patients with GMA

Pat. No.	Spontaneous HR	Anti-IgE	Control Allergen	Provocation allergen
1	1.7	5.4	3.1	8.7
2	2.6	12.6	11.5	17.3
3	3.4	5.2	4.5	39.1
4	3.2	6.2	-	8.8
5	3.3	6.0	3.1	3.0
6	0.5	3.7	0.9	5.1
7	4.1	1.6	8.9	27.6
8	7.5	2.9	8.3	3.2
9	7.0	6.6	4.9	8.1
10	2.5	3.6	3.0	4.8
11	0.3	3.6	-	1.5
12	1.4	7.9	2.4	11.3
13	5.3	-	3.7	11.2
14	22.4	25.6	20.7	57.9
15	25.8	23.0	-	28.7
16	1.5	-	-	1.8
17	4.8	9.3	-	6.7
18	2.9	3.8	2.8	5.8
19	0.1	1.1	0.4	2.4
Median:	3.20	5.40[b]	3.40	8.10[a]
(range)	0.1-25.8	1.1-25.6	0.4-20.7	1.5-57.9

Significance levels: [a]P = 0.008 *vs* spontaneous histamine release; [b]P = 0.04 *vs* spontaneous histamine release. HR: histamine release (average value from two measurements); no.: number; pat.: patient; anti-IgE: anti-immunoglobulin E. Control and provocation allergen are allergens, which were either tolerated or induced clinical symptoms during blinded oral food challenge procedures. Rates of histamine release are expressed as percentage (%) of total tissue histamine content. Each release value represents the average value from two repeat experiments (two separate biopsies).

Statistical analysis

From each patient, two rates of histamine release were obtained for each of the parameters spontaneous histamine release, anti-IgE, provocation and control allergen. The average value for each pair of release data was calculated and used for final statistics as listed in Table 3.

For descriptive statistics of the whole group, the median and range were chosen. Statistical comparisons were made using the U-test (Wilcoxon, Mann & Whitney) and significance levels are given in brackets. For comparison of the clinical tests, *ex vivo* biopsy stimulation by mucosa oxygenation was considered successful (positive) when the antigen containing solution caused an increase of the histamine release up to more than twice that of the spontaneous release.

RESULTS

Histamine release from colorectal tissue

Spontaneous histamine release from viable colorectal mucosal fragments amounted to only 3.2% of the total tissue histamine content, indicating that mast cells are able

Table 4 Colorectal mucosal histamine release in comparison with the outcome of oral provocation tests in patients with GMA

	Positive HR	Negative HR	Line sum
Positive BPCFC	12	7	19
Negative BPCFC	3	11	14
Column sum	15	18	

HR: histamine release, BPCFC: (double- or single) blinded, placebo-controlled oral food challenge. Colorectal mucosal histamine release: Sensitivity 63.1%, specificity 78.6%.

Table 5 Patients' history in comparison with the outcome of oral provocation tests in patients with GMA

	Positive H	Negative H	Line sum
Positive BPCFC	4	9	13
Negative BPCFC	3	4	7
Column sum	7	13	

H: patients' history, BPCFC: (double- or single) blinded, placebo-controlled oral food challenge. Note: Only 13 of 19 patients (68%) felt confident to answer questions concerning suspected provocation antigens, while the remaining were uncertain or had no experience of adverse reactions to the particular antigen. For evaluation of the specificity of patients' history, data from only 7 patients (37%) were available. Patients' history: Sensitivity 30.8%, specificity 57.1%.

Table 6 Skin prick tests in comparison with the outcome of oral provocation tests in patients with GMA

	Positive prick test	Negative prick test	Line sum
Positive BPCFC	9	10	19
Negative BPCFC	3	11	14
Column sum	12	21	

BPCFC: (double- or single) blinded, placebo-controlled oral food challenge Skin prick test: Sensitivity 47.4%; specificity 78.6%.

Table 7 Allergen-specific serum IgE determinations (RAST test) in comparison with the outcome of oral provocation tests in patients with GMA

	Positive RAST	Negative RAST	Line sum
Positive BPCFC	11	8	19
Negative BPCFC	7	7	14
Column sum	18	15	

BPCFC: (double- or single) blinded, placebo-controlled oral food challenge Allergen-specific IgE detection in serum: Sensitivity 57.9%; specificity 50.0%.

to maintain their normal metabolism and their mediators within granules during mucosa oxygenation (Table 3). Application of anti-human-IgE induced a clearly enhanced rate of histamine release of 5.4% (P = 0.04) within 30 min, confirming the functional reactivity of mucosal immune effector-and intestinal mucosal mast cells towards IgE-receptor cross-linking[15-17,21,23]. Four of 17 patients (23.5%) were found to be unresponsive to the anti-IgE concentrations used. Interestingly, these patients had the highest rates of spontaneous histamine release, possibly indicating that mast cells had already been degranulated or that a high rate of spontaneous histamine secretion may exert some negative feed-back mechanisms on mast cell triggering by anti-IgE (Table 3).

Incubation of intact colorectal tissue with BPCFC-negative food antigens did not induce a significant increase in histamine release (median increase 1.2 fold; range 0.6-4.4 fold of spontaneous mediator release) in patients with GMA. Histamine release with control antigens amounted to 3.4% and was not statistically different from spontaneous histamine release (Table 3).

In contrast, provocation allergens that evoked clinically significant reactions in allergic individuals (BPCFC-positive food antigens) already induced a 2.6 fold increased rate of histamine release compared to the spontaneous release (range 0.9-24 fold) during 30 min of mucosa oxygenation. The percentage of histamine release in response to provocation allergens was 8.1% and significantly different from spontaneous histamine release (P = 0.008).

Colorectal mucosal histamine release in comparison with established clinical tests

Colorectal mucosal histamine release was positive in 12 of 19 patients (sensitivity 63.1%, Table 4), who experienced a reproducible clinical reaction in response to the same provocation antigen. In contrast, 3 of 14 patients (21.4% false positive) discharged significant histamine amounts although oral provocation was negative. Control antigens, tolerated by the patient during BPCFC, were also found to be negative with regard to histamine release in 11 of 14 patients (specificity 78.6%).

When comparing established clinical parameters for food allergy diagnostics with the outcome from blinded provocation tests (gold standard), a lower diagnostic accuracy was obtained through reference to patients' history (Table 5), skin test results (Table 6) and allergen-specific IgE detection in serum (Table 7) than with histamine release experiments.

The comparison between patients' history and BPCFC (Table 5) was somewhat impeded, since only 13 of 19 patients (68.4%) knew their allergen inducing clinical symptoms, 7 of 14 patients (50%) with recurrent gastrointestinal complaints tried to give exact answers on questions about their well tolerated foods, while all other individuals had significant uncertainties about adverse reactions to or tolerance of the food antigens tested.

DISCUSSION

Diagnosis and existence of gastrointestinal food allergy are still a matter of debate[1,4,5,18]. To date, no exact diagnostic and practical relevant means are readily available for the gastroenterologist or endoscopist to examine the gastrointestinal mucosa for signs of food hypersensitivity, when patients with recurrent episodes of variable gastrointestinal complaints are referred for further diagnostics[5,8,10]. One innovative approach for the diagnosis of food hypersensitivity by endoscopy may be the use of a mucosa oxygenation system, which allows culturing of small viable endoscopic samples outside of

the patient for immunological release experiments[15-17,20,23]. Since histamine is one important and very early secreted mediator of different types of IgE and non-IgE mediated allergic reactions[1,3,17,24], this study was designed to evaluate colorectal mucosal histamine release from patients with gastrointestinal food hypersensitivity. In contrast to allergic individuals with typical extraintestinal signs of type I food hypersensitivity, diagnostic problems have been repeatedly reported in this patient population primarily involving the gastrointestinal tract with symptoms of allergic diarrhoea, vomiting, nausea, *etc*. In addition, the frequency of local gastrointestinal allergy in several important clinical conditions is also still unclear[1,4,5,8-11,25-27].

This study demonstrates that a significant histamine release can be induced from colorectal mucosa upon IgE receptor cross-linking or antigenic stimulation *ex vivo* by mucosa oxygenation using endoscopically taken samples[15-17,23]. This confirms that functional antigen-specific tests using histamine as the primary test parameter are equally feasible with mucosa from the lower gastrointestinal tract as from the upper gastrointestinal tract (duodenum[15]) or more long-lived mediators like mast cell tryptase or eosinophilic cationic protein[20,23]. However, in contrast to previously published work featuring tryptase or eosinophilic cationic protein, histamine release tests using bioptic tissue bear the distinct practical advantage that they can be performed within a short period of time of only 30 min, providing the appropriate technique to rapidly destroy all histamine catabolising enzymes in the drawn culture supernatants is applied[16,17,20,21]. The quick performance of mucosa oxygenation using histamine as a diagnostic parameter may qualify this test for its use in clinical practice, possibly as a refinement or complement of existing endoscopic-diagnostic procedures when patients with suspected gastrointestinal food allergy are being referred for diagnostic work-up.

Compared to other human tissues or isolated mast cells, histamine release from colorectal mucosa was found to be of a smaller magnitude than expected. This could be a result of the short cultivation period and of the fact that colorectal tissue harbours large quantities of histamine metabolising enzymes, which are still active within the viable cultured tissue at a physiological rate during mucosa oxygenation[6,11,17,20,21,28]. However, compared with the spontaneous rate of histamine release, functional mast cell stimulation by anti-IgE or BPCFC-positive antigens achieved a significantly higher rate of histamine release, while control antigens showed a similar degree of histamine release as the spontaneous secretion. This study proved unambiguously the reactivity of histologically normal gut mucosa in allergic patients upon specific challenge. In view of that, gastroenterologists will find a valuable addition to their diagnostic methods through the establishment of a functional test using biopsies of the involved and reacting allergic shock organ[15,21,23]. Depending on the number of biopsies taken during endoscopy, this functional test allows the simultaneous examination of different food antigens during mucosa oxygenation[15-17,21]. In this way, any contact between the antigen and the patient's immune system is avoided and the patient is not put at risk of any allergic symptoms or reactions.

Although only a small group of patients was investigated in this study, colorectal mucosal histamine release was found to yield a diagnostic sensitivity and specificity of 63.1% and 78.6%, while skin tests and serum IgE detection reached only a sensitivity and specificity of 47.3% and 78.6% or 57.5% and 50%, respectively. Similar low rates of diagnostic accuracy of the skin prick tests and RAST tests have also been reported by other investigators in GMA[1,2,4,5,12,25-27]. This may be explained by the fact that sensitisation in the cutaneous and serological compartment may not always accurately reflect the immunological response of the mucosal microenvironment at the large surface area of the gastrointestinal tract[1,18,25,27]. The presence of different (immunological) compartments with separate mechanisms for local IgE production in food hypersensitivity may also account for the known multitude of allergic manifestations in food allergy[1,8,29]. This is illustrated by the fact that several individuals in this study experienced significant clinical symptoms during BPCFC despite negative skin or serum IgE tests, confirming the need for further diagnostic means directly targeting the involved shock organ[1,5,25-27,29].

Mucosa oxygenation with histamine or more long-lived mediators bears the potential to predict the outcome of double-blind, placebo-controlled food challenges[17,21,23]. Hence, a future cost-effective approach of the gastroenterologist to diagnose gastrointestinal food allergy could possibly be based on the additional use of gut mucosal histamine release (Table 4) to avoid time-consuming, cost-intensive and sometimes risky challenge procedures. However, the real value of such a diagnostic procedure has yet to be established within greater patient populations[21,23]. The rationale of this proposal for future diagnostics is given by the fact that gut mucosal histamine release was proven to be of superior diagnostic value compared to that of the commonly used allergological means such as case history, skin prick tests or RAST tests, as these are not always concise or do not directly examine the involved organ in patients with GMA, respectively[1,2,25,27]. The data presented here suggest that patients with a food antigen-induced mucosal histamine release exceeding more than twice the amount of the spontaneous one be could directly subjected to a specific elimination diet.

As part of appropriately provided health care, prospective long-term analysis of the clinical course of these patients is always necessary. Diminishing financial resources, however, dictate the need for more economical investigations, which may be sufficed by mucosa oxygenation. Although this approach is more invasive than blinded food challenges, it needs only one endoscopic examination with testing of several antigens, while DBPCFC needs at least one (in-patient) test day for each individual allergen application. The data presented here suggest that mucosa oxygenation could perhaps eliminate the need to perform DBPCFC in a significant number of patients suspected of having GMA[16,17,21].

However, the fact that colorectal mucosal histamine release by mucosa oxygenation did not identify all BPCFC-positive allergens is more likely to be related to the

complex pathophysiology and compartmentalisation of gastrointestinal food allergy rather than to inadequately applied methods. Patients with positive provocation but negative colorectal histamine release, which was the case for 7 of 19 patients (36.8%), may develop their allergic reactions in more proximal parts of the gastrointestinal tract (e.g. stomach, duodenum). Or else, these patients may react only in blood or at extraintestinal sites, respond to the tested antigen mainly with other mediators rather than with histamine (e.g. arachidonic acid products, eosinophilic proteins *etc*) or may have produced the antigenic epitope after passage through the liver[1,5,8,25,29]. Conversely, control allergens, which elicited a significant histamine release in 3 of 14 patients (21.4%), may fail to provoke a clinically significant reaction unless histamine catabolism is sufficiently active[1,28]. In addition, the demonstration of food-specific histamine release from colorectal mucosa may also explain why some patients experience postprandial extraintestinal symptoms (urticaria, hypotension, asthma bronchiale *etc*) despite negative skin tests. In such cases, intestinally produced and released histamine may reach peripheral extraintestinal organs and activate their histamine receptors inducing classical extraintestinal allergic symptoms without the presence of food-specific IgE in the periphery. These different pathophysiological parameters in combination with several yet unknown or ill-defined factors (e.g. neurovegetative impulses, gut flora *etc*) contribute to or induce the great variability of clinical manifestations in GMA[1,3-5,10,18,23,29].

REFERENCES

1 **Crowe SE**, Perdue MH. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* 1992; **103**: 1075-1095

2 **Van Den Bogaerde J**, Cahill J, Emmanuel AV, Vaizey CJ, Talbot IC, Knight SC, Kamm MA. Gut mucosal response to food antigens in Crohn's disease. *Aliment Pharmacol Ther* 2002; **16**: 1903-1915

3 **Sampson HA**, Jolie PL. Increased plasma histamine concentrations after food challenges in children with atopic dermatitis. *N Engl J Med* 1984; **311**: 372-376

4 **Bengtsson U**, Nilsson-Balknäs U, Hanson LA, Ahlstedt S. Double blind, placebo controlled food reactions do not correlate to IgE allergy in the diagnosis of staple food related gastrointestinal symptoms. *Gut* 1996; **39**: 130-135

5 **Niggemann B**, Beyer K. Diagnostic pitfalls in food allergy in children. *Allergy* 2005; **60**: 104-107

6 **Prahl P**, Krasilnikof F, Stahl Skov P, Norn S. Basophil histamine release in children with adverse reactions to cow milk. Comparison with RAST and skin prick test. *Allergy* 1988; **43**: 442-448

7 **Soifer MM**, Hirsch SR. The direct basophil degranulation test and the intracutaneous test: a comparison using food extracts. *J Allergy Clin Immunol* 1975; **56**: 127-132

8 **Sampson HA**. Food allergy. Part 1: immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* 1999; **103**: 717-728

9 **Pollard H**, Stuart G. Experimental reproduction of gastric allergy in human beings with controlled observations on the mucosa. *J Allerg* 1942; **13**: 467-473

10 **Reimann HJ**, Ring J, Ultsch B, Wendt P. Intragastral provocation under endoscopic control (IPEC) in food allergy: mast cell and histamine changes in gastric mucosa. *Clin Allergy* 1985; **15**: 195-202

11 **Bischoff SC**, Mayer JH, Manns MP. Allergy and the gut. *Int Arch Allergy Immunol* 2000; **121**: 270-283

12 **Santos J**, Bayarri C, Saperas E, Nogueiras C, Antolín M, Mourelle M, Cadahia A, Malagelada JR. Characterisation of immune mediator release during the immediate response to segmental mucosal challenge in the jejunum of patients with food allergy. *Gut* 1999; **45**: 553-558

13 **Botoman VA**, Surawicz CM. Bacteremia with gastrointestinal endoscopic procedures. *Gastrointest Endosc* 1986; **32**: 342-346

14 **Kiss A**, Ferenci P, Graninger W, Pamperl H, Pötzi R, Meryn S. Endotoxaemia following colonoscopy. *Endoscopy* 1983; **15**: 24-26

15 **Baenkler HW**, Lux G. Antigen-induced histamine-release from duodenal biopsy in gastrointestinal food allergy. *Ann Allergy* 1989; **62**: 449-452

16 **Raithel M**, Hochberger J, Baenkler HW. [Allergic enteropathy. Intestinally-mediated fungus allergy]. *Fortschr Med* 1991; **109**: 582-585

17 **Raithel M**, Matek M, Baenkler HW, Jorde W, Hahn EG. Mucosal histamine content and histamine secretion in Crohn 's disease, ulcerative colitis and allergic enteropathy. *Int Arch Allergy Immunol* 1995; **108**: 127-133

18 **Schwab D**, Raithel M, Klein P, Winterkamp S, Weidenhiller M, Radespiel-Troeger M, Hochberger J, Hahn EG. Immunoglobulin E and eosinophilic cationic protein in segmental lavage fluid of the small and large bowel identify patients with food allergy. *Am J Gastroenterol* 2001; **96**: 508-514

19 **Weidenhiller M**, Müller S, Schwab D, Hahn EG, Raithel M, Winterkamp S. Microscopic (collagenous and lymphocytic) colitis triggered by food allergy. *Gut* 2005; **54**: 312-313

20 **Raithel M**, Hochberger J, Hahn EG. Effect of colonoscopy premedication containing diazepam and pethidine on the release of mast cell mediators in gut mucosal samples. *Endoscopy* 1995; **27**: 415-423

21 **Bijlsma PB**, Backhaus B, Weidenhiller M, Donhauser N, Hahn EG, Raithel M. Food allergy diagnosis by detection of antigen-induced electrophysiological changes and histamine release in human intestinal biopsies during mucosa-oxygenation. *Inflamm Res* 2004; **53** Suppl 1: S29-S30

22 **Morel AM**, Delaage MA. Immunoanalysis of histamine through a novel chemical derivatization. *J Allergy Clin Immunol* 1988; **82**: 646-654

23 **Schwab D**, Müller S, Aigner T, Neureiter D, Kirchner T, Hahn EG, Raithel M. Functional and morphologic characterization of eosinophils in the lower intestinal mucosa of patients with food allergy. *Am J Gastroenterol* 2003; **98**: 1525-1534

24 **Rangachari PK**. Histamine: mercurial messenger in the gut. *Am J Physiol* 1992; **262**: G1-13

25 **Aiuti F**, Paganelli R. Food allergy and gastrointestinal diseases. *Ann Allergy* 1983; **51**: 275-280

26 **Paajanen L**, Vaarala O, Karttunen R, Tuure T, Korpela R, Kokkonen J. Increased IFN-gamma secretion from duodenal biopsy samples in delayed-type cow's milk allergy. *Pediatr Allergy Immunol* 2005; **16**: 439-444

27 **André F,** André C, Colin L, Cavagna S. IgE in stools as indicator of food sensitization. *Allergy* 1995; **50**: 328-333

28 **Raithel M**, Ulrich P, Keymling J, Hahn EG. Analysis and topographical distribution of gut diamine oxidase activity in patients with food allergy. *Ann N Y Acad Sci* 1998; **859**: 258-261

29 **Teitelbaum JE**, Furuta GT. Immune mechanisms of food allergy. *Curr Opin Gastroenterol* 1998; **14**: 498-503

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CLINICAL RESEARCH

Correlation of *p53* gene mutation and expression of P53 protein in cholangiocarcinoma

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Abstract

AIM: To characterize the tumor suppressor gene *p53* mutations and study the correlation of *p53* gene mutation and the expression of P53 protein in cholangiocarcinoma.

METHODS: A total of 36 unselected, frozen samples of cholangiocarcinoma were collected. *p53* gene status(exon 5-8) and P53 protein were examined by automated sequencing and immunohistochemical staining, combined with the clinical parameters of patients.

RESULTS: *p53* gene mutations were found in 22 of 36 (61.1%) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between the positive and negative expression of P53 protein. However, there were no significant differences in pathologic parameters between the mutations and non-mutations.

CONCLUSION: The alterations of the *p53* gene evaluated by DNA sequence analysis is relatively accurate. Expression of P53 protein could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

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Key words: Cholangiocarcinoma; *p53* gene; Mutation; DNA sequence

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INTRODUCTION

Tumor suppressor gene, besides oncogene, is involved in the development of cancer, which inhibits cell proliferation and formation of tumor. Normally tumor suppressor gene counteracts with oncogene to protect an organism against cancer. The *p53* tumor suppressor gene is the most common mutated gene in human cancer[1-9], occurring in approximately 50% cancers[10-12]. Cholangiocarcinoma is among the most common malignant tumors. Mutation of *p53* is one of the most frequently encountered genetic alterations in cholangiocarcinoma. *p53* mutations play a central role in carcinogenesis of cholangiocarcinoma.

Alterations of the *p53* gene can be evaluated by DNA sequencing analysis and immunohistochemistry(IHC). P53 protein overexpression is considered a poor prognosis of cholangiocarcinoma[13-17]. But IHC has shown controversial results over that of DNA sequencing analysis. We examined mutation status of *p53* gene by automated sequencing and IHC in 36 cases of cholangiocarcinoma and studied the correlation of *p53* gene mutation with clinical parameters and its clinical significance.

MATERIALS AND METHODS

Patients

A total of 36 unselected, frozen samples were obtained from patients with cholangiocarcinoma who had been treated by surgical resection from April 2000 to May 2005 in the Department of General Surgery of the First Affiliated Hospital of China Medical University and Hepatobiliary Surgery of the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College. The types of cholangiocarcinoma included 18 cases of tubular adenocarcinomas, 9 cases of papillary adenocarcinomas, 4 cases of mucoid carcinomas and 5 cases of undifferentiated carcinoma. Among them, well-moderately differentiated was 25 and poorly differentiated was 11 cases respectively. There were 16 cases of T1 stage, 10 cases of T2 stage and 10 cases of T3 stage by the UICC standard. Lymph node metastasis was seen in 33 cases. Non- lymph node metastasis was seen in 3 cases. The patients contained 23 males

Table 1 Primers sequences of p53 gene exons 5-8

Primers	Sequences	
	Sense	Anti-sense
Exon 5	5′-TGT TCA CTT GTG CCC TGA CT-3′	5′-CAG CCC TGT CGT CTC TCC AG-3′
Exon 6	5′-ACA GGG CTG GTT GCC CAG GGT-3′	5′-CTC CCAGAG ACC CCA GTT GC-3′
Exon 7	5′-GGT CTC CCC AAG GCG CAC TGG-3′	5′-AGG CTG GGG GCA CAG CAG GCC-3′
Exon 8	5′-ATT TCC TTA CTG CCT CTT GC-3′	5′-AAG TGA ATC TGA GGC ATA AC-3′

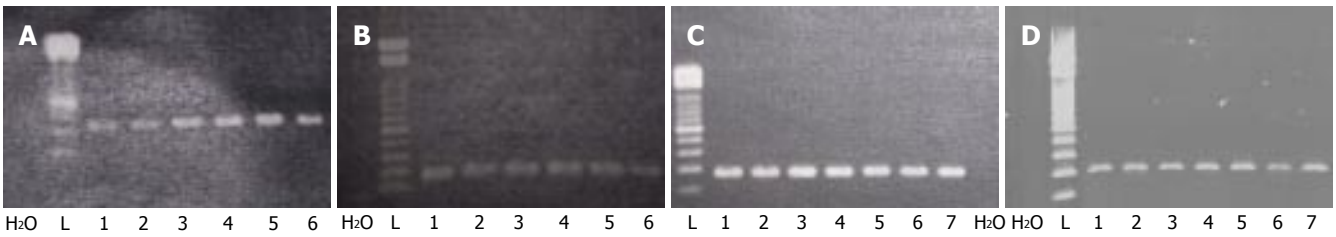

Figure 1 *p53* exons 5, 6, 7, 8 PCR product. L : 100 bp DNA ladder; 1-7: Samples. **A**: Exons 5; **B**: Exons 6; **C**: Exons 7; **D**: Exons 8.

and 13 females, with age ranging from 36 to 71 (median, 61.2) years. All of the samples were frozen at -80℃ until DNA extraction and subjected to histological diagnosis by a pathologist.

DNA extraction

DNA was extracted from tissues using a QIAamp DNA Micro kit: QIA (Germany). Tissue samples weighing less than 10 mg were placed into a 1.5 mL microcentrifuge tube. Immediately 180 μL buffer ATL and 20 μL proteinase K were added and mixed by pulse-vortexing for 15 s. Then they were incubated at 56℃ overnight. Two hundred microliter buffer AL and 200 μL ethanol (100%) were added and incubated for 5 min at room temperature. After that, all of the lysates were applied onto the QIAamp MinElute column. Five hundred microliter buffer AW1 and buffer AW2 were added. After centrifugation, 100 μL buffer AE was applied to get DNA. DNA quantity was determined by the ratio of A260/280.

Primer sequences and PCR amplification

Table 1 shows primer sequences used for *p53* exons 5-8, which was synthesized by Hokkaido Bioscience Co. (Japan). PCR used a 20 μL reaction volume containing 1 unit of Hot start EXTaq DNA polymerase (Takara, Biochemical, Japan), 2 μL of 10 × EXTaq buffer, 2 μL of dNTP mixture and each primer (8 pmol each for reaction) and 1 μL of DNA template. The condition of the first PCR is as follows: 96℃ for 3 min for denaturation, 40 cycles of 96℃ for 30 s, 60℃ for 30 s, 72℃ for 30 s with a final elongation step of 4 min at 72℃. Water was used as a negative control. Five microliters of PCR product were analyzed on 1% TBE gel electrophoresis. Each sample was repeated three times.

DNA sequencing

All of the PCR products were purified using Auto seq TMG-50 (Amersham Biochemical Company, USA). BigDye Terminator Cycle sequencing Ready Reaction (Perkin Elmer, USA) was used. The primers of sequencing were the same as PCR primers. But its concentration was one tenth of PCR primers. The condition is as follows: 95℃ for 4 min, 95℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s for 40 cycles with a final step of 4 min at 72℃. Both sense and antisense chains were analyzed on an ABI prism 310 Genetic Analyzer (Perkin Elmer). Each sample was repeated three times.

Immunohistochemistry

Five-micron sections were dewaxed in xylene and rehydrated. Endogenous peroxidase was destroyed by a 15-min treatment in 30 mL/L hydrogen peroxide(H_2O_2) in phosphate-buffered saline (PBS) at room temperature. The sections were blocked with a combination of normal mouse serum and then incubated with anti-p53 protein(dilution 1:50, mouse anti-p53 protein by Boster Co.), followed by biotinylated-conjugated sheep anti-mouse IgG (Boster Co.). The complex was visualized by diaminobenzidine(Boster Co.). The specificity of the reaction was confirmed by use of negative control, blank control and substitution control, in which PBS substituted the secondary antibody biotinylated-conjugated sheep anti-mouse IgG.

Statistical analysis

The results were analyzed with χ^2 test. $P < 0.05$ was taken as significant.

RESULTS

p53 gene mutations and expression of P53 protein in cholangiocarcinoma tissues

Positive band of exons 5-8 was found in all samples after PCR amplification (Figures 1 A-D). *p53* gene mutations were detected in 22 of 36 patients (61.1%) by DNA sequencing. Among them, there were 7 cases of exon 5 mutations, which were located on 161,175 and 196 codons. All were transition (G:C/A:T). Six cases of exon 6 mutations were located on 209, 213 and 215 codons, of which 4 cases were of transition(G:C/A:T) and 2 cases

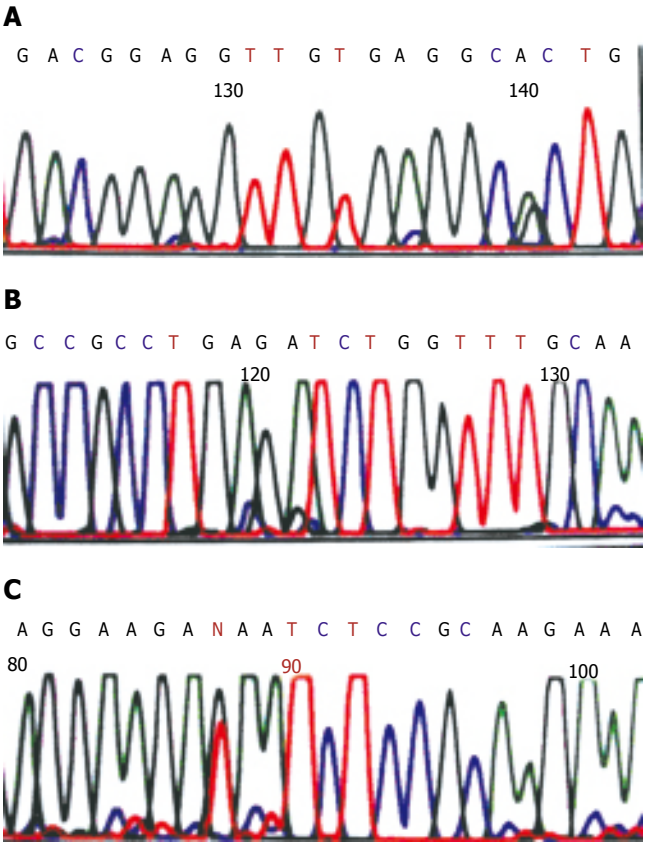

Figure 2 Tranversion of *p53* exons 5, 6 , 8 in cholangiocarcinoma. **A**: Exons 5 (G: C/A: T); **B**: Exons 6 (G: C/A: T); **C**: Exons 8 (G→T).

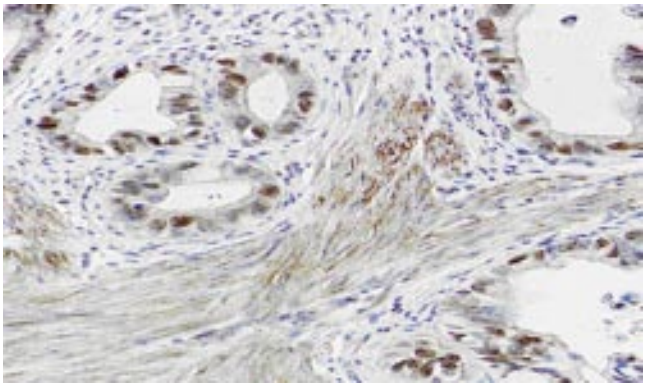

Figure 3 P53 protein was positive in cholangiocarcinoma (SABC × 400).

of tranversion (G-T). Three cases of exon 7 mutations were located on 248, 252 codons. Six cases of exon 8 mutations were located on 282, 287, 289, 306 codons, of which 4 cases were of transition (G:C/A:T) and 2 cases of tranversion (G-T) (Figures 2 A-C).

Nineteen cases (52.8%) were positive for P53 protein. P53 protein localized in the nuclei of cholangic epithelial cells. Moreover, it was crisp and finely granular (Figure 3).

Correlation of p53 gene mutation and clinical parameters

There were significant differences in degree of differentiation and invasion between the positive and negative samples of P53 protein expression ($P < 0.05$, $P < 0.05$). However, there were no significant differences in age, gender, degree of differentiation and invasion, lymph node metastasis, stage between the mutations and non-mutations ($P > 0.05$) (Table 2).

Table 2 Correlation of mutation status of *p53* gene and P53 protein expression with pathologic parameters in cholangiocarcinoma

Pathologic character	*n*	*p53* gene mutation Positive (*n*, %)	*P* vaule	P53 protein expression Positive (*n*, %)	*P* vaule
Pathologic type					
Tubular carcinoma	18	11 (61.1)		10 (55.6)	
Papillary carcinoma	9	6 (66.7)		5 (55.6)	
Mucoid carcinoma	4	2 (50.0)		2 (50.0)	
Non-differentiated carcinoma	5	3 (60.0)	> 0.05	2 (40.0)	> 0.05
Differentiation					
Well -Moderate	25	13 (52.0)		9 (36.0)	
Poor	11	9 (81.8)	> 0.05	10 (90.9)	< 0.05[a]
Invasion					
T1	16	7 (43.8)		2 (12.5)	
T2	10	8 (80.0)		8 (80.0)	
T3	10	7 (70.0)	> 0.05	9 (90.0)	< 0.05[c]
Lymph node metastasis					
N0	3	2 (66.7)		0 (0)	
N1	33	20 (60.6)	> 0.05	19 (57.5)	> 0.05

DISCUSSION

Cholangiocarcinoma is the second most common cancer of the hepatobiliary system. In recent years, the incidence and mortality of cholangiocarcinoma have been increasing in China[18,19]. In most patients, the disease is only diagnosed at a late stage. Patients with obstructive jaundice are frequently at the advanced stage of the disease, which is contraindicated for operation[20-26]. The development of molecular biology, the identification of molecular factors involved in cholangiocarcinoma carcinogenesis, and the elucidation of the mechanisms will significantly impact prevention, diagnosis, treatment and prognosis. *p53* gene is located on the short arm of chromosome 17, which consists of 11 exons. There are four mutation hot-spots(132-143, 174-179, 236-248, 272-281) within the core domain (exon 5-8), which are the key sites of biological activity of P53 protein[27,28]. *p53* gene is an important regulator factor of cell proliferation. It is related to cell cycles, DNA repair, cell differentiation and apoptosis. In the presence of DNA damage the expression of *p53* is enhanced and induces G1 cell cycle arrest until DNA is repaired. If repair is insufficient, *p53* gene promotes apoptotic cell death. However, *p53* gene mutation cannot block cell proliferation. Through cooperation with inactivation of tumor suppressor genes and activation of oncogenes, cells transform into malignant ones and become a tumor. *p53* tumor suppressor gene is the most common mutated gene in human cancer and is frequently seen in cholangiocarcinoma. Recent studies have found that positive expression of P53 protein is related to invasion and lymph node metastasis in cholangiocarcinoma[15,16].

p53 gene mutation could act as an index to estimate the prognosis of cholangiocarcinoma.

We examined mutation status of *p53* gene exons 5-8 by automated sequencing in 36 cases of cholangiocarcinoma. We found *p53* gene mutations in 22 of 36 (61.1 %) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between positive and negative expression of P53 protein. However, there were no significant differences in age, gender, extent of differentiation, invasion, lymph node metastasis, and stage between the mutations and non-mutations.

Wild-type *p53* (non-mutated) has a short half-life of about 20 min. Mutant P53 protein has a greater stability with half-life prolonged up to 1.4-7 h. It can be detected by IHC method. But the use of different P53 antibodies and methods can result in a marked difference in the degree of overexpression, and varying levels of overexpression may also be noted in the same tumor specimen. IHC has been shown to have discordancy rates of 30%-35% compared with DNA sequencing. Thus, the determination of *p53* overexpression is not an accurate measure of *p53* function. Although DNA sequence analysis is a cumbersome, time-consuming and difficult method on archived material, it could provide a more accurate means of detecting *p53* mutations. Thus, we think *p53* gene mutation could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

REFERENCES

1 **Forslund A**, Kressner U, Lönnroth C, Andersson M, Lindmark G, Lundholm K. P53 mutations in colorectal cancer assessed in both genomic DNA and cDNA as compared to the presence of *p53* LOH. *Int J Oncol* 2002; **21**: 409-415

2 **Gasco M**, Shami S, Crook T. The *p53* pathway in breast cancer. *Breast Cancer Res* 2002; **4**: 70-76

3 **Staib F**, Hussain SP, Hofseth LJ, Wang XW, Harris CC. TP53 and liver carcinogenesis. *Hum Mutat* 2003; **21**: 201-216

4 **Caldeira S**, Filotico R, Accardi R, Zehbe I, Franceschi S, Tommasino M. *p53* mutations are common in human papillomavirus type 38-positive non-melanoma skin cancers. *Cancer Lett* 2004; **209**: 119-124

5 **Baroni TE**, Wang T, Qian H, Dearth LR, Truong LN, Zeng J, Denes AE, Chen SW, Brachmann RK. A global suppressor motif for *p53* cancer mutants. *Proc Natl Acad Sci USA* 2004; **101**: 4930-4935

6 **Lin JT**, Wang JS, Jiann BP, Yu CC, Tsai JY, Huang JK, Wu TT. Correlation of *p53* protein accumulation and Bcl-2 overexpression with histopathological features in prostatic cancer. *J Formos Med Assoc* 2005; **104**: 864-867

7 **El Far MA**, Atwa MA, Yahya RS, El Basuni MA. Evaluation of serum levels of *p53* in hepatocellular carcinoma in Egypt. *Clin Chem Lab Med* 2006; **44**: 653-656

8 **Saetta AA**. K-ras, *p53* mutations, and microsatellite instability (MSI) in gallbladder cancer. *J Surg Oncol* 2006; **93**: 644-649

9 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. *World J Gastroenterol* 2006; **12**: 192-198

10 **Harms K**, Nozell S, Chen X. The common and distinct target genes of the *p53* family transcription factors. *Cell Mol Life Sci* 2004; **61**: 822-842

11 **Mir MM**, Dar NA, Gochhait S, Zargar SA, Ahangar AG, Bamezai RN. *p53* mutation profile of squamous cell carcinomas of the esophagus in Kashmir (India): a high-incidence area. *Int J Cancer* 2005; **116**: 62-68

12 **Moreno M**, Pimentel F, Gazdar AF, Wistuba II, Miquel JF. TP53 abnormalities are frequent and early events in the sequential pathogenesis of gallbladder carcinoma. *Ann Hepatol* 2005; **4**: 192-199

13 **Wang X**, Zhang J, Chen J. [Result of *p53*, ki-67 protein expression in cholangiocarcinoma with in situ hybridization and immunohistochemistry methods]. *Zhongguo Yixue Kexueyuan Xuebao* 2000; **22**: 57-60

14 **Ahrendt SA**, Rashid A, Chow JT, Eisenberger CF, Pitt HA, Sidransky D. *p53* overexpression and K-ras gene mutations in primary sclerosing cholangitis-associated biliary tract cancer. *J Hepatobiliary Pancreat Surg* 2000; **7**: 426-431

15 **Horie S**, Endo K, Kawasaki H, Terada T. Overexpression of MDM2 protein in intrahepatic cholangiocarcinoma: relationship with *p53* overexpression, Ki-67 labeling, and clinicopathological features. *Virchows Arch* 2000; **437**: 25-30

16 **Ito Y**, Takeda T, Sasaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Matsuura N. Expression and clinical significance of the G1-S modulators in intrahepatic cholangiocellular carcinoma. *Oncology* 2001; **60**: 242-251

17 **Attallah AM**, Abdel-Aziz MM, El-Sayed AM, Tabll AA. Detection of serum *p53* protein in patients with different gastrointestinal cancers. *Cancer Detect Prev* 2003; **27**: 127-131

18 **Jan YY**, Yeh CN, Yeh TS, Chen TC. Prognostic analysis of surgical treatment of peripheral cholangiocarcinoma: two decades of experience at Chang Gung Memorial Hospital. *World J Gastroenterol* 2005; **11**: 1779-1784

19 **Liu XF**, Zhou XT, Zou SQ. An analysis of 680 cases of cholangiocarcinoma from 8 hospitals. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 585-588

20 **Reddy SB**, Patel T. Current approaches to the diagnosis and treatment of cholangiocarcinoma. *Curr Gastroenterol Rep* 2006; **8**: 30-37

21 **Singh P**, Patel T. Advances in the diagnosis, evaluation and management of cholangiocarcinoma. *Curr Opin Gastroenterol* 2006; **22**: 294-299

22 **Mansfield SD**, Barakat O, Charnley RM, Jaques BC, O' Suilleabhain CB, Atherton PJ, Manas D. Management of hilar cholangiocarcinoma in the North of England: pathology, treatment, and outcome. *World J Gastroenterol* 2005; **11**: 7625-7630

23 **Patel T**. Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 33-42

24 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314

25 **Sakata J**, Shirai Y, Wakai T, Nomura T, Sakata E, Hatakeyama K. Catheter tract implantation metastases associated with percutaneous biliary drainage for extrahepatic cholangiocarcinoma. *World J Gastroenterol* 2005; **11**: 7024-7027

26 **Hubert C**, Sempoux C, Berquin A, Deprez P, Jamar F, Gigot JF. Bile duct carcinoid tumors: an uncommon disease but with a good prognosis? *Hepatogastroenterology* 2005; **52**: 1042-1047

27 **Giglia-Mari G**, Sarasin A. TP53 mutations in human skin cancers. *Hum Mutat* 2003; **21**: 217-228

28 **Khan SA**, Taylor-Robinson SD, Carmichael PL, Habib N, Lemoine NR, Thomas HC. Analysis of *p53* mutations for a mutational signature in human intrahepatic cholangiocarcinoma. *Int J Oncol* 2006; **28**: 1269-1277

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RAPID COMMUNICATION

Growth hormone regulates intestinal ion transport through a modulation of the constitutive nitric oxide synthase-nitric oxide-cAMP pathway

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Abstract

AIM: Growth hormone (GH) directly interacts with the enterocyte stimulating ion absorption and reducing ion secretion induced by agonists of cAMP. Since nitric oxide (NO) is involved in the regulation of transepithelial ion transport and acts as a second messenger for GH hemodynamic effects, we tested the hypothesis that NO may be involved in the resulting effects of GH on intestinal ion transport.

METHODS: Electrical parameters reflecting transepithelial ion transport were measured in Caco-2 cell monolayers mounted in Ussing chambers and exposed to GH and cholera toxin (CT) alone or in combination, in the presence or absence of the NO synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME). Similar experiments were conducted to determine cAMP and nitrite/nitrate concentrations. NOS expression was assayed by Western blot analysis.

RESULTS: L-NAME causes total abrogation of absorptive and anti-secretory effects by GH on intestinal ion transport. In addition, L-NAME was able to inhibit the GH-effects on intracellular cAMP concentration under basal conditions and in response to CT. GH induced a Ca^{2+}-dependent increase of nitrites/nitrates production, indicating the involvement of the constitutive rather than the inducible NOS isoform, which was directly confirmed by Western blot analysis.

CONCLUSION: These results suggest that the GH effects on intestinal ion transport, either under basal conditions or in the presence of cAMP-stimulated ion secretion, are mediated at an intracellular level by the activity of cNOS.

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Key words: Nitric oxide synthase; Cholera toxin; Intestinal ion secretion; cAMP; Enterocyte

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INTRODUCTION

Intestinal ion fluxes are regulated by several agents including neurotransmitters, hormones, or paracrine agents[1]. We obtained evidence that growth hormone (GH) and nitric oxide (NO) act as modulators in this network[2-5]. GH increases basal intestinal water and ion absorption in *in vivo* and *in vitro* animal models and is also capable of substantially reducing ion secretion induced by agonists of cAMP, cGMP, or intracellular Ca^{2+}, the second messengers of ion secretion[4,5]. Using the human intestinal cell line Caco-2, we showed that the GH effects on ion transport result from direct interaction with the enterocyte[2]. Free radical NO acts as a second messenger of several GH effects on human metabolism[6]. NO production is decreased in patients with untreated GH deficiency, while treatment with recombinant human growth hormone (rhGH) increases NO formation[7]. In the past decade NO has emerged as a signalling molecule mediating a broad spectrum of intestinal processes, such as gastrointestinal motility, inflammatory changes, malignancy, mucosal blood flow and transepithelial ion transport[8,9]. NO is a gas with a half life of less then 5 s generated through a series of regulated electron transfer steps by a family of P450-like enzymes, termed nitric oxide synthases (NOS)[10,11]. Two NOS are continuously present and are termed constitutive nitric oxide synthase (cNOS). These two isoforms are Ca^{2+}/calmodulin-dependent, produce small amounts of NO in

short bursts and are involved in homeostatic processes. A third isoform, which is Ca^{2+}/calmodulin-independent, is induced by intestinal injury and inflammation. This latter isoform, termed inducible nitric oxide synthase (iNOS), requires a lag period of at least 2-3 h and, once expressed, produces large amounts of NO for longer time[13,12]. NO can be directly produced by enterocytes through both the constitutive and the inducible NOS isoforms[3,13,14]. An important feature of the NO effect is its concentration-dependence. Leading to the concept that NO often acts as a double-edged sword mediator with beneficial as well as detrimental effects. While at lower concentrations it maintains a basal ion intestinal pro-absorptive tone, it increases in several pathologic states such as inflammatory bowel diseases, toxic megacolon, and infectious gastroenteritis, contributing to ion secretion[8,12,15]. Recently, we showed that under basal conditions the intracellular cAMP concentration ([cAMP]i) is downregulated in the enterocyte by a cNOS-dependent NO production. Furthermore, in the presence of a cAMP-dependent stimulated secretion, cNOS is activated functioning as a breaking force of ion secretion[3]. This raised the hypothesis that the enterocyte is capable of self-regulating its own ion transport process through the activation of the cNOS-NO pathway which is able to modulate the [cAMP]i level[3]. The aim of this study was to determine whether NO is also involved in mediating the ion absorptive effects triggered by an extracellular stimulus. Specifically, we tested the hypothesis that the cNOS-NO-cAMP pathway is implicated in the pro-absorptive and in the anti-secretory effect induced by GH at the intestinal level. We used the Caco-2 in vitro cell model, previously validated for investigating the GH and the NO intestinal effects[2].

MATERIALS AND METHODS

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose concentration (4.5 g/L) supplemented with 10% FCS, 1% nonessential amino acids, penicillin (50 mU/mL), streptomycin (50 mg/mL) and were incubated in 50 mL/L CO_2-950 mL/L air. Medium was changed daily.

Ion transport studies

Cells were grown on uncoated polycarbonate transwell filters as previously described and used for intestinal transport studies at 15 d post-confluence[3]. The filter area was 4.9 cm^2. Each filter was mounted in an Ussing chamber (World Precision Instrument, Sarasota, FL) as a flat sheet between the mucosal and the serosal compartment. Each compartment contained 10 mL of Ringer's solution with the following composition (in mmol/L): NaCl (114), KCl (10), Na_2HPO_4 (1.65), NaH_2PO_4 (0.3), $CaCl_2$ (1.25), $MgCl_2$ (1.1), $NaHCO_3$ (15), glucose (19). In experiments performed to investigate the role of Cl^- in the electrical response, SO_4^- substituted Cl^- at an equimolar concentration. The incubation fluid was circulated by a thermostat-regulated circulating pump and continuously gassed with 95% O_2-5% CO_2. Transepithelial potential difference (PD), short-circuit current (Isc) and tissue ionic conductance (G) were monitored by an automatic voltage-clamp device (DVC 1000, World Precision Instrument, Sarasota, FL) as described elsewhere[16], before and after mucosal or serosal addition of GH, cholera toxin (CT), and the specific NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Isc is expressed as microamperes per square centimeter (μA/cm^2), G as millisiemens per square centimeter (mS/cm^2), and PD as millivolts (mV). Caco-2 cell monolayers, pre-incubated for 20 min with GH (4×10^{-9} mol/L) on the serosal side, were exposed to CT (6×10^{-8} mol/L) on the mucosal side, in the presence or the absence of L-NAME (2×10^{-4} mol/L) added to both sides. The maximal effective concentrations of GH, CT and L-NAME were determined by dose-response experiments (data not shown). Cell viability was evaluated at the end of each experiment by measuring the electrical response to the serosal addition of theophylline (5×10^{-3} mol/L).

Intracellular cAMP concentration determination

To test the hypothesis that GH specifically counteracts the CT-induced cAMP increase, we determined the modifications in [cAMP]i after 1 h of incubation with GH and CT, alone or in combination, and in the presence or the absence of L-NAME. [cAMP]i in Caco-2 cells which was measured by using a commercial kit (Biotrak cyclic AMP assay system; Amersham International, Amersham, UK), as previously described[17].

Western blot analysis

Caco-2 cells were stimulated with GH (4×10^{-9} mol/L) for 1, 6 or 24 h. Cells were then scraped into PBS buffer and lysed in a buffer containing 1% Tergitol (Nonidet P-40) with the following composition: KCl, 60 mmol/L; β-mercaptoethanol, 14 mmol/L; EDTA, 2 mmol/L; HEPES pH 7.9, 15 mmol/L; sucrose, 0.3 mol/L; aprotinin, 5 μg/mL; leupeptin, 10 μg/mL; pepstatin, 2 μg/mL; phenylmethylsulfonyl fluoride, 0.1 mmol/L. Whole cellular extracts were centrifuged at 1500 g for 20 m at 4℃. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Munchen, Germany). The supernatant containing the solubilized proteins was then boiled for 5 min in Laemly buffer (tris-HCl pH 6.8, 62.5 mmol/L; SDS 2%; glycerol 10%; 2-mercaptoethanol 5%; bromophenol blue 0.001%). Cell proteins (50 μg/lane) were added to an SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (BioBlot-NC-Costar, Corning Incorporated, Canada). Blots were blocked with T-TBS buffer (tris-HCl pH 8.8, 10 mmol/L; NaCl, 150 mmol/L; Tween 20, 0.05%) containing 3% albumin, and probed for 1 h with affinity purified anti-human NOS1 (1:2000) dilution ratio, NOS2 (1:200) or NOS3 (1:1000) rabbit polyclonal antibodies. Bound antibody was detected with anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody and developed by chemiluminescence reaction (Amersham Pharmacia Biotech, UK). Gamma-interferon (50 000 U/mL) was used as a positive control in experiments performed using anti-iNOS antibodies.

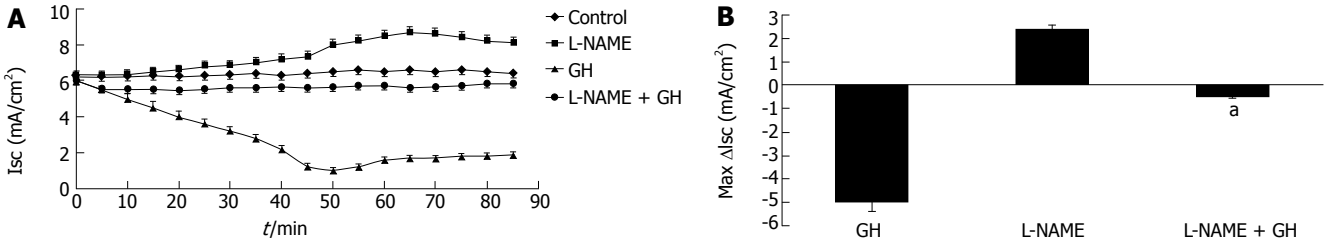

Figure 1 **A**: Time course of the effect on short-circuit current (Isc) of GH (4×10^{-9} mol/L) and L-NAME (2×10^{-4} mol/L), alone or in combination, to Caco-2 cells mounted in Ussing chambers; **B**: Isc peak after L-NAME or GH addition, alone or in combination, to Caco-2 cells mounted in Ussing chambers. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ GH *vs* L-NAME + GH.

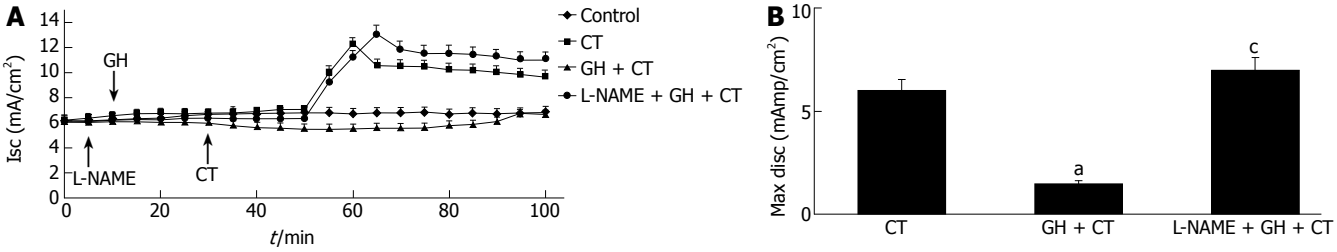

Figure 2 **A**: Time course of the GH (4×10^{-9} mol/L) effect on CT (6×10^{-8} mol/L)-induced short-circuit a current (Isc) increase in the absence or in the presence of L-NAME (2×10^{-4} mol/L) in Caco-2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent; **B**: Maximal Isc increase after CT addition, alone or in the presence of GH alone or in combination with L-NAME. A total abrogation of the antagonistic effect of GH on the CT-induced electrical response was observed in the presence of L-NAME. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ CT alone *vs* GH + CT; $^{c}P < 0.05$ GH + CT *vs* L-NAME + GH + CT.

Nitrite/nitrate (NO_2^-/NO_3^-) production

The combined concentration of nitrite and nitrate, the degradation products of NO in the culture medium, was determined by the Griess reaction after nitrate reduction[18]. Total nitrite/nitrate production was referred to NO production. Experiments were performed using normal or Ca^{2+}-free Ringer's solution to investigate the involvement of the cNOS isoform (the Ca^{2+}/calmodulin-dependent isoform or NOS1). The modified Ringer's solution had the following composition (mmol/L): $NaHPO_4$, 1.65; NaH_2PO_4, 0.3; $NaHCO_3$, 15, NaCl, 53; KCl, 10; Na_2SO_4, 30.5; $MgCl_2$, 2.35; glucose, 19; EDTA, 0.5.

Chemicals

All chemicals were of reagent grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Culture media were from Life Technologies GIBCO BRL (Mascia e Brunelli, Milan, Italy). Transwell filters and supports were from Costar (Costar Italia, Milan, Italy). rhGH was obtained from Serono (Industria Farmaceutica Serono, Rome, Italy). Anti-cNOS and anti-iNOS polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-iNOS polyclonal antibodies were purchased from Transduction Laboratories (ABD Company, Lexington, KY, USA).

Statistical analysis

Each experiment was run in duplicate and repeated at least 3 times. Results are expressed as mean ± SD. Repeated-measures ANOVA were applied using the Bonferroni test for multiple comparisons. The significance was set at 5%. The SPSS software package for Windows (release 11.0.1; SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Intestinal transport studies

GH (4×10^{-9} mol/L) and L- NAME (2×10^{-4} mol/L) caused opposite effects on basal Isc. GH induced a decrease and L-NAME induced an increase in Isc. Both effects were totally related to PD modifications, as no significant variations of G were recorded. Pre-treatment with L-NAME (2×10^{-4} mol/L) for 5 m almost abolished the electrical response to GH (Figure 1). The addition of CT (6×10^{-8} mol/L) to Caco-2 cells induced an increase in Isc. Both the GH and CT effects were Cl^--dependent as demonstrated in the experiment done in Cl^- free Ringer solution. Thus, in the absence of Cl^- the electrical effects were virtually abolished indicating that they were entirely due to transepithelial Cl^- transport modifications (data not shown). Pre-incubation with GH for 20 m substantially reduced the CT effect on Isc. However, pre-incubation with L-NAME resulted in total abrogation of the antagonistic effect of GH on the CT-induced electrical response (Figure 2).

Intracellular cAMP concentration determination

Incubation with GH (4×10^{-9} mol/L) resulted in significant reduction of basal [cAMP]i. On the contrary, incubation with CT induced an increase in [cAMP]i. The addition of L-NAME produced a significant increase in basal and in CT-stimulated [cAMP]i. Pre-incubation with GH for 20 min resulted in a reduction of the CT-induced [cAMP]i increase. Finally, the addition of L-NAME resulted in total inhibition of the GH-induced decrease in basal [cAMP]i as

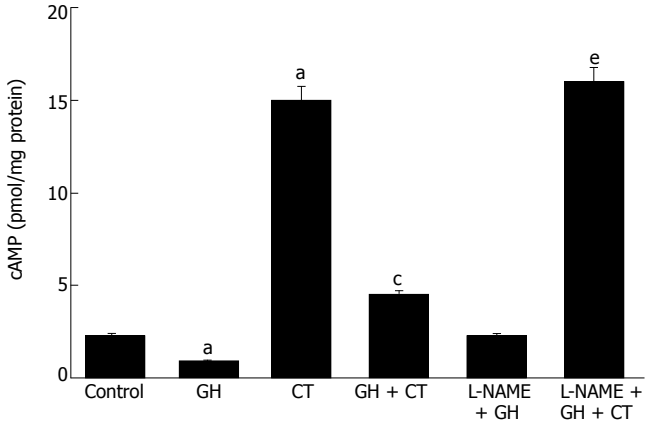

Figure 3 Modification of intracellular cAMP concentration in Caco-2 cells after incubation with GH, CT, alone or in combination, in the presence or in the absence of L-NAME. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ CT alone *vs* GH + CT; $^{e}P < 0.05$ GH + CT *vs* L-NAME + GH + CT.

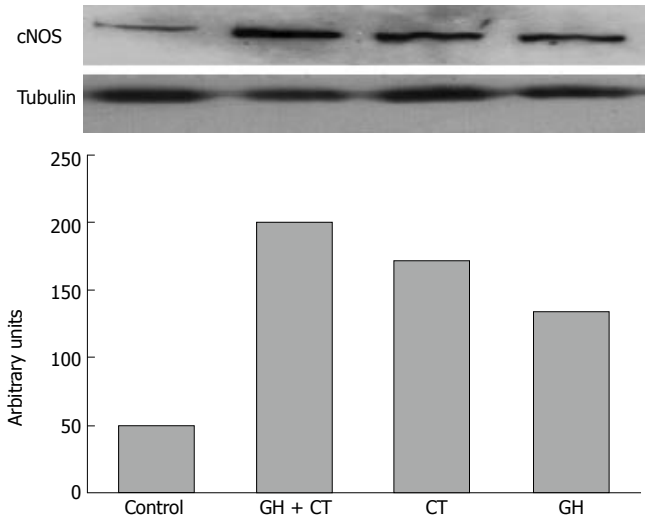

Figure 4 The upper side of the figure shows the cNOS protein expression in Caco-2 cells after 1 h of incubation with GH and CT alone or in combination, as compared to tubulin expression. The cNOS protein expression is revealed by the appearance of 160-kD band that corresponds to human NOS 1 (neuronal NOS). Shown is a representation of 3 separate experiments. In the lower side of the figure an optical densitometry analysis of the bands is also reported.

well as a total abrogation of the GH effect on CT-induced [cAMP]i increase (Figure 3). These results point to a role of NO either under basal conditions or in response to external stimuli which drive ion fluxes toward an absorption pattern.

Western blot analysis

Caco-2 cells showed low but detectable basal cNOS protein expression (Figure 4). Western blot analysis performed after 1 h of incubation with GH revealed the amplification of a 160 kD band corresponding to human NOS 1. Simultaneous incubation of Caco-2 cells with either GH or CT resulted in further amplification of the NOS 1 band (Figure 4). On the contrary, NOS 2 and NOS 3 protein expressions were undetectable in unstimulated cells and in cells exposed to GH and CT alone or in combination for up to

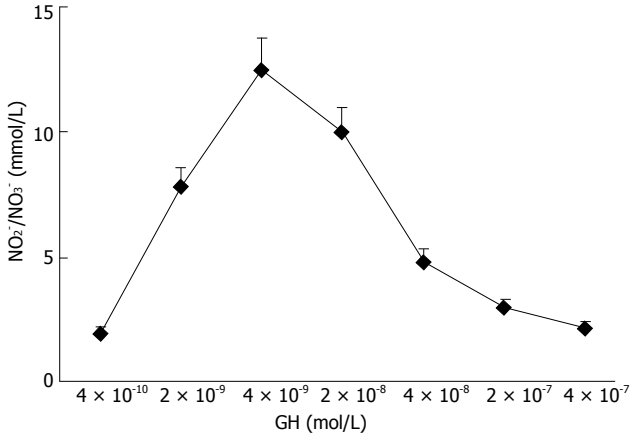

Figure 5 Effects of increasing concentration of GH on NO production in Caco-2 cells. Increasing concentrations of GH were added to Caco-2 cell monolayers and NO production was determined after 1 h of incubation. Data are mean ± SD of 6 different observations.

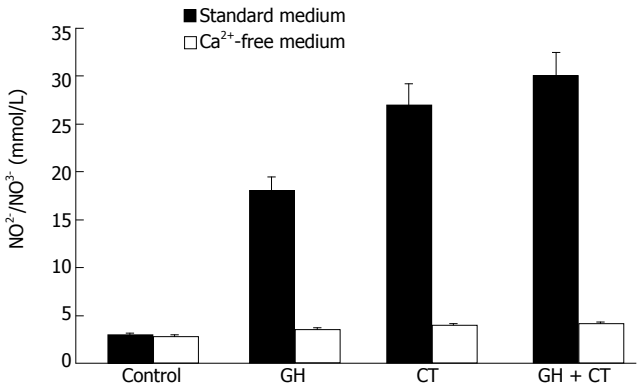

Figure 6 Total NO production in Caco-2 cells under basal conditions and after stimulation with GH and CT, alone or in combination, in standard or in Ca^{2+}-free medium. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ *vs* control.

24 h of incubation (data not shown).

Nitrite/nitrate (NO_2^-/NO_3^-) production

NO production by Caco-2 cells was determined in culture medium after 1 h of incubation with increasing GH doses. As shown in Figure 5 a dose-dependent increase in NO production was detected in response to the hormone. GH doses higher than 4×10^{-9}mol/L did not induce further increase in NO production, indicating a saturation pattern of the effect. Caco-2 cell stimulation with simultaneous exposure to GH and CT resulted in a further increase in NO production compared to each individual substance. The effect was Ca^{2+}-dependent, since in the absence of Ca^{2+}, the NO increase in response to GH addition was abolished. This data suggests an involvement of the constitutive rather than the inducible NOS isoform in the GH effect (Figure 6).

DISCUSSION

We have previously shown that GH is able to increase intestinal fluid absorption under basal conditions and to inhibit ion secretion elicited by the 3 main intracellu-

lar second messengers of bacterial enterotoxins: cAMP, cGMP and Ca^{2+}[2,4]. The data from this study provides new evidence on the ability of GH to regulate water and ion transport and implicates cNOS-NO activity for this effect. A complete abrogation of GH effects on Isc was seen in the presence of the specific NOS inhibitor L-NAME. An increase of cNOS activity and a subsequent Ca^{2+}-dependent production of NO were observed in enterocytes treated with GH. These effects were associated with a Cl^--dependent decrease in Isc, consistent with an anion absorptive effect. We have recently demonstrated that the CT-enterocyte interaction results in an enhanced NO production. Such an effect may be interpreted as a homeostatic mechanism operated by the enterocyte and functioning as a breaking force to limit ion secretion[3]. cNOS-NO system is activated by a yet unknown sensing mechanism and reacts to balance the stimulated secretion. The data in this work support and extend this hypothesis and suggest that the modulation of the cNOS-NO activity could also be dependent on extracellular stimuli control, namely on the GH signal transduction. In all instances, the target of cNOS-NO is cAMP. Our results are similar to those obtained using NO donors, which are able to inhibit forskolin-stimulated cAMP production by adenyl cyclase (AC) isoforms AC5 and AC6, in both T84 epithelial cells and mucosal scrapings from mouse colon[19-21], and are in agreement with those previously obtained in isolated cholangiocytes[22].

It has been recently suggested that GH inhibitory effect on intestinal ion secretion is related to the transactivation of epidermal growth factor (EGF) receptor and the subsequent activation of extracellular signal-regulated kinase (ERK, also known as p 44/42 mitogen activated protein kinase or MAPK) activity[23]. Interestingly, a NO stimulation through ERK-dependent upregulation of cNOS gene transcription has been recently demonstrated for proinsulin C-peptide in endothelial cells[24], and similar effects have been shown with the endothelium-derived hyperpolarizing factor (EDHF)[25]. Thus, it is possible to also hypothesize that the NO-mediated GH effects at the intestinal level could involve a MAPK activation. It also remains to be clarified whether GH effects are mediated by Ca^{2+}. Overall the cNOS-NO system could be viewed as a regulator of ion transport acting on the enterocyte via three distinct patterns: (1) to keep cAMP production at a low level under basal conditions, in order to maintain an intestinal ion pro-absorptive tone; (2) during stimulation of ion secretion, such as that trigged by CT, to reduce ion secretion; (3) in response to extracellular pro-absorptive stimuli, namely acting as second messenger of the GH-induced ion absorption. In all these 3 instances the target of cNOS-NO is cAMP, the effect is Ca^{2+}-dependent and involves Cl^- transcellular flux. Thus the cNOS-NO-cAMP pathway plays a key role on the enterocyte fluid absorptive/secretory processes.

REFERENCES

1 **Hansen MB**, Skadhauge E. New aspects of the pathophysiology and treatment of secretory diarrhoea. *Physiol Res* 1995; **44**: 61-78

2 **Canani RB**, Bisceglia M, Bruzzese E, Mallardo G, Guarino A. Growth hormone stimulates, through tyrosine kinase, ion transport and proliferation in human intestinal cells. *J Pediatr Gastroenterol Nutr* 1999; **28**: 315-320

3 **Canani RB**, Cirillo P, Buccigrossi V, De Marco G, Mallardo G, Bruzzese E, Polito G, Guarino A. Nitric oxide produced by the enterocyte is involved in the cellular regulation of ion transport. *Pediatr Res* 2003; **54**: 64-68

4 **Guarino A**, Canani RB, Iafusco M, Casola A, Russo R, Rubino A. In vivo and in vitro effects of human growth hormone on rat intestinal ion transport. *Pediatr Res* 1995; **37**: 576-580

5 **Berni Canani R**, Iafusco M, Russo R, Bisceglia M, Polito G, Guarino A. Comparative effects of growth hormone on water and ion transport in rat jejunum, ileum, and colon. *Dig Dis Sci* 1996; **41**: 1076-1081

6 **Campbell GS**. Growth-hormone signal transduction. *J Pediatr* 1997; **131**: S42-S44

7 **Böger RH**. Nitric oxide and the mediation of the hemodynamic effects of growth hormone in humans. *J Endocrinol Invest* 1999; **22**: 75-81

8 **Izzo AA**, Mascolo N, Capasso F. Nitric oxide as a modulator of intestinal water and electrolyte transport. *Dig Dis Sci* 1998; **43**: 1605-1620

9 **Cirino G**. Nitric oxide releasing drugs: from bench to bedside. *Dig Liver Dis* 2003; **35** Suppl 2: S2-S8

10 **Bredt DS**, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 1991; **351**: 714-718

11 **Stark ME**, Szurszewski JH. Role of nitric oxide in gastrointestinal and hepatic function and disease. *Gastroenterology* 1992; **103**: 1928-1949

12 **Fasano A**. Nitric oxide and intestinal water and electrolyte transport: in, out, or both? *J Pediatr Gastroenterol Nutr* 2001; **32**: 527-528

13 **Blachier F**, Darcy-Vrillon B, Sener A, Duée PH, Malaisse WJ. Arginine metabolism in rat enterocytes. *Biochim Biophys Acta* 1991; **1092**: 304-310

14 **Tepperman BL**, Brown JF, Whittle BJ. Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am J Physiol* 1993; **265**: G214-G218

15 **Schirgi-Degen A**, Beubler E. Involvement of K+ channel modulation in the proabsorptive effect of nitric oxide in the rat jejunum in vivo. *Eur J Pharmacol* 1996; **316**: 257-262

16 **Field M**, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. *Am J Physiol* 1971; **220**: 1388-1396

17 **Guarino A**, Bisceglia M, Canani RB, Boccia MC, Mallardo G, Bruzzese E, Massari P, Rappuoli R, Telford J. Enterotoxic effect of the vacuolating toxin produced by Helicobacter pylori in Caco-2 cells. *J Infect Dis* 1998; **178**: 1373-1378

18 **Moore WM**, Webber RK, Jerome GM, Tjoeng FS, Misko TP, Currie MG. L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem* 1994; **37**: 3886-3888

19 **Freeman SL**, MacNaughton WK. Ionizing radiation induces iNOS-mediated epithelial dysfunction in the absence of an inflammatory response. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G243-G250

20 **Asfaha S**, Bell CJ, Wallace JL, MacNaughton WK. Prolonged colonic epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. *Am J Physiol* 1999; **276**: G703-G710

21 **Freeman SL**, MacNaughton WK. Nitric oxide inhibitable isoforms of adenylate cyclase mediate epithelial secretory dysfunction following exposure to ionising radiation. *Gut* 2004; **53**: 214-221

22 **Spirlì C**, Fabris L, Duner E, Fiorotto R, Ballardini G, Roskams T, Larusso NF, Sonzogni A, Okolicsanyi L, Strazzabosco M. Cytokine-stimulated nitric oxide production inhibits adenylyl cyclase and cAMP-dependent secretion in cholangiocytes. *Gastroenterology* 2003; **124**: 737-753

23 **Chow JY**, Carlstrom K, Barrett KE. Growth hormone reduces

chloride secretion in human colonic epithelial cells via EGF receptor and extracellular regulated kinase. *Gastroenterology* 2003; **125**: 1114-1124

24 **Kitamura T**, Kimura K, Makondo K, Furuya DT, Suzuki M, Yoshida T, Saito M. Proinsulin C-peptide increases nitric oxide production by enhancing mitogen-activated protein-kinase-dependent transcription of endothelial nitric oxide synthase in aortic endothelial cells of Wistar rats. *Diabetologia* 2003; **46**: 1698-1705

25 **Wang H**, Lin L, Jiang J, Wang Y, Lu ZY, Bradbury JA, Lih FB, Wang DW, Zeldin DC. Up-regulation of endothelial nitric-oxide synthase by endothelium-derived hyperpolarizing factor involves mitogen-activated protein kinase and protein kinase C signaling pathways. *J Pharmacol Exp Ther* 2003; **307**: 753-764

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RAPID COMMUNICATION

Low utility of plasma Nociceptin/orphanin FQ in the diagnosis of hepatocellular carcinoma

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Abstract

AIM: The utility of serum alpha-fetoprotein (α-FP) in the detection of hepatocellular carcinoma (HCC) is questionable. Very high circulating levels of nociceptin/orphanin FQ (N/OFQ), a ligand for a novel opioid receptor, have recently been reported in HCC. The aim of this study was to assess the role of plasma N/OFQ in the diagnosis of HCC arising in patients with liver cirrhosis.

METHODS: Plasma N/OFQ levels were measured by ELISA in 58 patients (28 HCC and 30 liver cirrhosis) and in 25 healthy controls. The values were correlated with clinical and laboratory features including α-FP. Spearman index, biserial correlation coefficient, non parametric combination (NPC) test and discriminant stepwise analysis were used for statistical evaluation of data.

RESULTS: The upper normal limit of nociceptin was 122 pg/mL. Plasma levels above this cut-off were found in 21.4% of patients with HCC, in 23.3% of those with cirrhosis and in 8% of healthy subjects. α-FP serum levels > 200 ng/mL were found in 46.4% of the patients with HCC and in none of those with cirrhosis. No correlation was found between N/OFQ levels and any of the clinical and laboratory features, including α-FP. By NPC test, HCC and cirrhotic patients were different with regard to α-FP (P = 0.000) but not in terms of nociceptin (P = 0.595). By point biserial correlation, HCC presence was positively correlated with α-FP (rpb = 0.52, P = 0.000) but not with N/OFQ (rpb = 0.16, P = 0.157). In a discriminant analysis, α-FP was significant in the Wilks test (Y = -0.709 + 0.03 α-FP) and properly classified 81% of all patients and 61% of HCC. N/OFQ had lower sensitivity, specificity and predictive values than α-FP.

CONCLUSION: Nociceptin is increased in patients with chronic liver disease, independently of the presence of HCC, although the underlying mechanism has yet to be clarified. We conclude it is not a useful marker for HCC.

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Key words: Hepatocellular carcinoma; Nociceptin/orphanin FQ; Liver cirrhosis; Alpha-fetoprotein

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INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is a 17-aminoacid neuropeptide with selective binding affinity for a novel opioid receptor-like 1 (ORL1/NOP). N/OFQ is synthesized in the neurons of the central and peripheral nervous system and is present in the blood and in the cerebrospinal fluid. The N/OFQ/NOP system has been implicated in a variety of biological functions at both central and peripheral levels: pain modulation, immunity, memory, learning, feeding, locomotion, thermoregulation and activities of the gastrointestinal, cardiovascular, renal and respiratory systems[1,2]. However, its precise biological role in humans has not been fully defined. The presence of N/OFQ/NOP has also been reported in human neuroblastoma cell-lines[3]. N/OFQ is detectable in the blood and high circulating levels have been reported in incidences of acute and chronic pain, where they are correlated with the duration of symptoms[4]. In Wilson disease, probably in relation to altered catabolism caused by liver and/or brain deposits of copper[5], and in cirrhosis of the liver[6] slightly increased circulating levels of N/OFQ have recently been found. Moreover, very high

levels have been reported in patients with hepatocellular carcinoma (HCC) suggesting that plasma N/OFQ level might represent a specific marker for HCC[6].

HCC is the leading cause of death in patients with cirrhosis of the liver[7]. The most commonly used serological marker for HCC surveillance, alpha-fetoprotein (α-FP), has highly variable sensitivity and specificity[8] while the diagnostic utility of other markers, such as des-γ-carboxyprothrombin[9], human hepatocyte growth factor[10], and serum chromogranin-A[11] remains unclear.

The aim of this study was to investigate the utility of plasma N/OFQ levels in the diagnosis of HCC in patients with liver cirrhosis.

MATERIALS AND METHODS

Patients and healthy subjects

Fifty-eight consecutively observed patients, 28 with HCC and 30 with liver cirrhosis, and 25 healthy members of the medical staff were included in the study. Informed written consent was obtained from patients and healthy controls. The diagnosis of HCC was based on imaging findings and/or histological confirmation. The diagnosis of liver cirrhosis was clinical and/or histological. The demographic and clinical characteristics of the populations studied are shown in Table 1.

Parameters examined

Clinical features (age, gender, etiology, Child-Pugh score, HCC size, presence of pain) and laboratory parameters as reported in Table 2 (plasma N/OFQ, serum α-FP, blood glucose, blood urea, serum creatinine, K, Na, Ca, bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, lactic dehydrogenase, creatine phosphokinase, amylases, cholinesterase, albumin, erythrocyte sedimentation rate, C-reactive protein, fibrinogen, international normalized ratio, hemoglobin, red blood cells, white blood cells, platelets) were evaluated through statistical analysis.

N/OFQ assay

Blood drawn from fasting subjects between 7.30 and 9.30 AM was collected in tubes containing K-EDTA. Aprotinin was immediately added to inhibit proteases. Plasma samples, stored at -80℃ for less than one month, were loaded onto C-18 containing Sep-columns (Phoenix Europe GmbH, Karlsruhe, Germany), washed with 0.1% trifluoroacetic acid, eluted with 60% acetonitrile in 1% trifluoroacetic acid and freeze-dried. ELISA assay of N/OFQ was performed using a commercially available kit (Phoenix Europe GmbH, Karlsruhe, Germany). Briefly, the lyophilized samples reconstituted with assay buffer were placed in microwells, together with rabbit anti-orphanin FQ serum and biotinylated peptide. After 2 h incubation at room temperature, streptavidin-horseradish peroxidase was added followed by a 1 h incubation. After washing, 100 μL of substrate solution was added and the reaction was terminated after 1 h with 100 μL of 2N HCl. Absorbance was read at 450 nm and results were compared to a standard curve ranging from 10 to 100.000 pg/mL.

Table 1 Clinical-Demographic characteristics of the populations studied

	HCC	Cirrhosis	Controls
Number of patients	28	30	25
Mean age ± SD (yr)	70 ± 4.8	65.3 ± 11.1	35.4 ± 13.9
Male/Female	20/8	21/9	10/15
Etiology *n* (%)			
HCV	24 (85.8)	15 (50)	
HBV	-	3 (10)	
Alcohol	2 (7.1)	8 (26.7)	
Cryptogenic	2 (7.1)	4 (13.3)	
Child – Pugh score *n* (%)			
A	22 (78.6)	19 (63.3)	
B	6 (21.4)	7 (23.3)	
C		4 (13.3)	
Patients with pain (%)	4 (14.3)	5 (16.6)	6 (24)
HCC diameter min-max (cm)	1.2-12.5		

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

Statistical analysis

Correlations among the variables studied were calculated using non-parametric Spearman index and biserial correlation coefficient[12], as appropriate. Differences between groups were evaluated using the Non Parametric Combination Test, NPC[13]. $P < 0.05$ was considered as statistically significant. Variables discriminating the groups of patients were identified with discriminant stepwise analysis (Wilks test and Classification results)[14]. The upper normal limit of N/OFQ was defined as the 95th percentile of the values in healthy controls. Software packages used were Methodologica S.R.L. (2001) for non-parametric analysis NPC test, Confidence Interval Analysis (CIA) Windows version 2.0 (2000) for sensitivity and specificity evaluation, and SPPS, Windows 11.0 (2001) for correlation index and discriminant analysis.

RESULTS

The upper normal limit of N/OFQ was 122 pg/mL. Plasma levels above this cut-off were found in 6 patients with HCC (21.4%), in 7 patients with cirrhosis (23.3%) and in 2 healthy subjects (8%). α-FP serum levels > 200 ng/mL, reported in the literature as highly suggestive for HCC[20], were found in 13 patients with HCC (46.4%) and in none of the patients with cirrhosis (Figure 1).

In all the groups, N/OFQ levels were not correlated with any of the demographic-clinical features nor with the laboratory parameters mentioned in the materials and methods section. In particular, N/OFQ levels were not correlated with α-FP levels.

The NPC test showed that patients with HCC and patients with cirrhosis were significantly different with regard to α-FP ($P = 0.000$), but not according to N/OFQ levels ($P = 0.595$). Point biserial correlation showed that HCC presence was positively correlated with the levels of α-FP ($rpb = 0.52$ $P = 0.000$) but not with those of N/OFQ ($rpb = 0.16$ $P = 0.157$). Within the discriminant

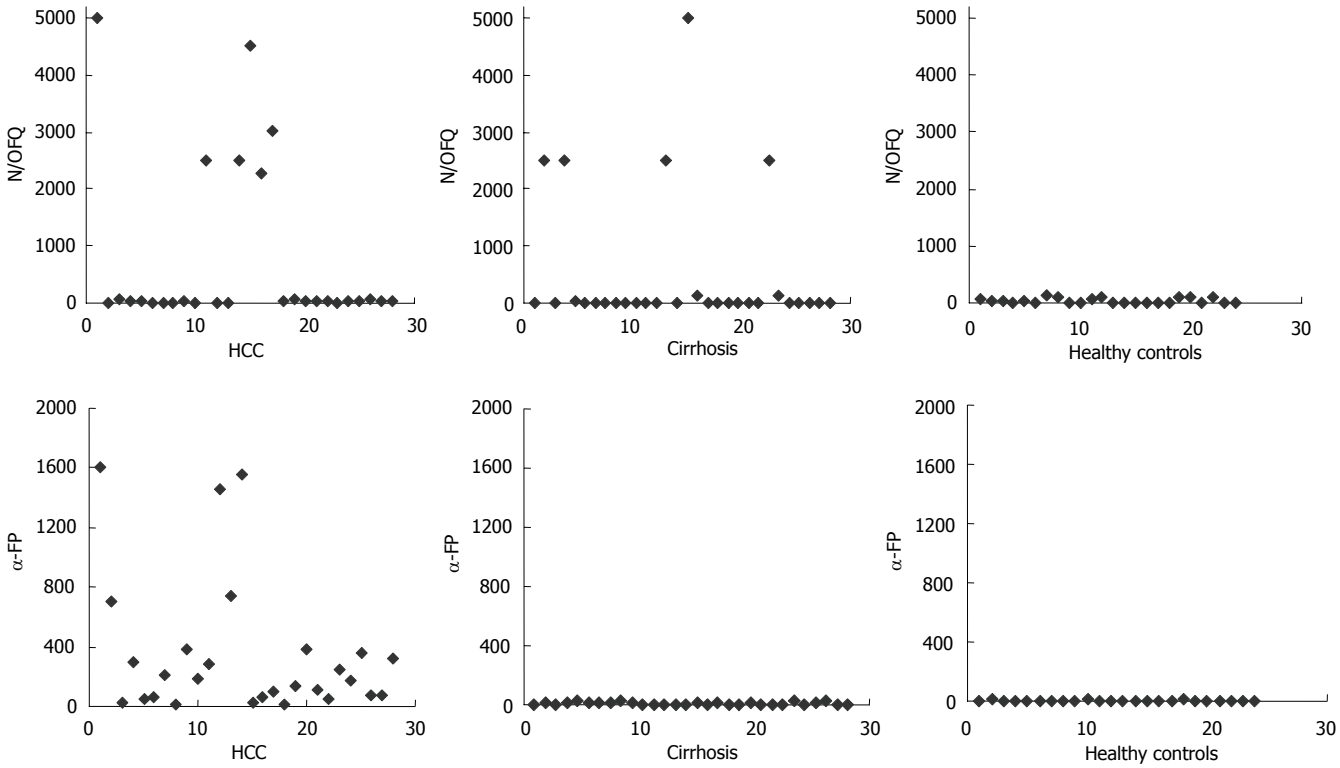

Figure 1 N/OFQ (pg/mL) and α-FP (ng/mL) values in patients with HCC, with cirrhosis and in healthy controls.

Table 2 Laboratory parameters included in the statistical analysis

Mean values ± SD	HCC	Cirrhosis	Controls
α-FP (ng/mL)	346 ± 461[b]	9.2 ± 8	3.9 ± 0.4
N/OFQ (pg/mL)	728 ± 1460	515 ± 1205	138 ± 493
Aspartate aminotransferase (U/L)	60 ± 28.5	54 ± 35	26 ± 9
Alanine aminotransferase (U/L)	37 ± 15	53 ± 50.5	33 ± 10.5
γ-glutamyl transpeptidase (U/L)	111 ± 58	78 ± 60	28 ± 11
Alkaline phosphatase (U/L)	325 ± 113.5	285 ± 185	122 ± 75
Cholinesterase (U/L)	3695 ± 1910	4105 ± 2891	9680 ± 2899
Bilirubin (mg/dL)	1.6 ± 1.3	3.7 ± 7	0.5 ± 0.3
Albumin (g/dL)	3.2 ± 0.4	3.4 ± 0.6	3.7 ± 0.4
Lactic dehydrogenase (U/L)	418 ± 53.5	347 ± 89	263 ± 78
Creatine phosphokinase (U/L)	136 ± 61	72 ± 18	87 ± 52
Amylases (U/L)	107 ± 19	90 ± 33	76 ± 45
Blood urea (mg/dL)	44 ± 16	71 ± 42.5	32 ± 11
Creatinine (mg/dL)	1.1 ± 0.3	1.2 ± 0.5	0.9 ± 0.2
Na (mmol/L)	135 ± 3.8	136 ± 6.8	139 ± 5.1
K (mmol/L)	4.4 ± 0.3	4.5 ± 0.5	4.3 ± 0.5
Ca (mg/dL)	9.1 ± 0.5	8.7 ± 0.4	4.9 ± 0.3
Red blood cells (10^6/mm^3)	3.8 ± 0.6	1.9 ± 1.7	4.2 ± 0.2
With blood cells (10^3/mm^3)	4.9 ± 2	5 ± 1.8	7 ± 1.3
Hemoglobin (g%)	11.7 ± 1.5	10.6 ± 2.2	13.6 ± 0.9
Platelets (10^3/mm^3)	116 ± 48	99 ± 47	249 ± 58
Blood glucose (mg/dL)	96 ± 14	111 ± 38	78 ± 11
Erythrocyte sedimentation rate (mm)	66.6 ± 17.7	49.4 ± 17.1	7.4 ± 4.1
C-reactive protein (mg/L)	18.4 ± 29.7	10.3 ± 9.7	2.7 ± 1.4
Internationl Normalized Ratio	1.22 ± 0.2	1.31 ± 0.3	0.8 ± 0.1
Fibrinogen (mg/L)	288 ± 103	243 ± 68	291 ± 93

[b]$P < 0.001$ *vs* cirrhosis and controls.

Table 3 Diagnostic accuracy tests

	N/OFQ		α-FP	
Sensitivity % (CI)	21.4	(0.06 - 0.36)	46.4	(0.29 - 0.64)
Specificity % (CI)	76.7	(0.61 - 0.91)	100	(0.88 - 1.00)
Positive predictive value % (CI)	46.2	(0.19 - 0.73)	100	(0.77 - 1.00)
Negative predictive value % (CI)	51.1	(0.36 - 0.65)	66.7	(0.52 - 0.78)
Positive likelihood ratio (CI)	0.92	(0.35 - 2.40)	∞	(4.01 - ∞)
Negative likelihood ratio (CI)	1.02	(0.77 - 1.35)	0.53	(0.33 - ∞)

analysis, the Wilks test showed that only α-FP was significant in the model (Y = -0.709 + 0.03 α-FP). The classification of results demonstrated that α-FP properly classified 81% of all patients and 61% of patients with HCC. Sensitivity, specificity and predictive values of N/OFQ and α-FP in the diagnosis of HCC are reported in Table 3.

DISCUSSION

HCC is the third leading cancer-related cause of death worldwide[15]. In western countries, HCC arises in cirrhotic livers with an annual incidence of 3%-5%. Early detection of HCC allows optimal application of curative treatments. The increased survival, after radical treatment in the last decade, recommends surveillance for detection of early HCC in cirrhosis[16]. Ultrasonography plays a key role in the detection of HCC but its sensitivity for small

nodules is low[17] and other imaging techniques, such as CT and MRI, are too expensive for screening programs. An elevation in serum α-FP is associated with HCC but it is also elevated in non-hepatic malignancies[18] and in hepatitis[19]. Although the diagnostic accuracy of serum α-FP is highly variable in the reported series depending on the cut-off level[20], it remains the most commonly used serological marker for HCC surveillance. The diagnostic value of other markers proposed so far, such as des-γ-carboxyprothrombin[9], human hepatocyte growth factor[10], serum chromogranin-A[11], is not well defined. It has recently been published that plasma levels of N/OFQ are very high in all patients with HCC, including those with normal α-FP[6]. With the aim to define if plasma N/OFQ values represent an indicator of HCC even in absence of increased α-FP, we studied circulating N/OFQ and α-FP levels in patients with liver cirrhosis, with and without HCC, comparing the diagnostic value of the two markers.

Mean N/OFQ values were higher both in patients with liver cirrhosis alone and in those with HCC than in controls, but did not differ significantly between the two patient groups, with a similar percentage of values above the normal range. On the other hand, mean α-FP levels in HCC patients were higher than in cirrhotics, with a higher percentage of values > 200 ng/mL. The NPC test showed significant differences between patients with and without HCC with regard to α-FP but not for N/OFQ. Such data are in accordance with the results of Szalay *et al*[6] with respect to the increase in plasma N/OFQ values seen in cirrhotic patients, but do not confirm the unique finding of extremely elevated values in the entire population with HCC. Since the demographic and clinical features, including size of the tumor and presence of pain, are similar among the patients enrolled in the two studies, we are not able to suggest any explanation other than possible genetic differences for the discordant results.

Receptors for N/OFQ are normally present in the liver as shown by detection of mRNA for the ORL1/NOP receptor[21]. Therefore, an increase in plasma N/OFQ values in some patients with chronic liver disease, with and without HCC, might be due to the chronic liver disorder with a reduced ability to bind N/OFQ.

Transcription of the N/OFQ gene is enhanced by estrogen[22], therefore another explanation for the increase of N/OFQ plasma levels in cirrhotic livers, independently of the presence of HCC, might be the increase of estrogen hormones, common in patients with advanced liver disease.

A higher N/OFQ content has been shown in HCC tissue as compared to tumor-free liver tissue in one patient with PBC[23] and in rats with experimentally induced tumors[24], suggesting that the HCC cells might produce N/OFQ or give signals for neuronal production. However, we were not able to find any correlation between plasma N/OFQ levels and tumor size, in agreement with the finding of Szalay *et al*[6].

Peripheral blood neutrophils express and secrete N/OFQ following degranulation[25]; N/OFQ stimulates chemotaxis[26] and is present at sites of inflammation, such as synovial exudates[25]. However, in this study, we were not able to correlate the amounts of circulating N/OFQ with any of the inflammatory indices (erythrocyte sedimentation rate, C-reactive protein, peripheral leukocytes, fibrinogen, platelets).

It has been demonstrated that N/OFQ plays a role in the perception of pain, inhibiting the release of various neurotransmitters, including pain related peptides[27,28]. However, no correlation between plasma values of N/OFQ and the presence of pain in any of the groups studied was found.

When we compared the diagnostic utility of N/OFQ and α-FP in HCC, α-FP had better sensitivity (46% *vs* 21%), better specificity (100% *vs* 77%), higher positive predictive value (100% *vs* 46%) and negative predictive value (67% *vs* 51%).

In conclusion, from our experience, N/OFQ cannot be recommended as a marker for early detection of HCC in patients with liver cirrhosis. α-FP is very specific when a high cut-off (200 ng/mL) is adopted but its sensitivity is low. N/OFQ is increased in patients with chronic liver disease, independent of the presence of HCC, although the underlying mechanism needs to be clarified. In the diagnosis of HCC, further research is needed in order to find serum markers more sensitive than α-FP.

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REFERENCES

1 **Calo' G**, Guerrini R, Rizzi A, Salvadori S, Regoli D. Pharmacology of nociceptin and its receptor: a novel therapeutic target. *Br J Pharmacol* 2000; **129**: 1261-1283

2 **Mogil JS**, Pasternak GW. The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol Rev* 2001; **53**: 381-415

3 **Spampinato S**, Di Toro R, Qasem AR. Nociceptin-induced internalization of the ORL1 receptor in human neuroblastoma cells. *Neuroreport* 2001; **12**: 3159-3163

4 **Ko MH**, Kim YH, Woo RS, Kim KW. Quantitative analysis of nociceptin in blood of patients with acute and chronic pain. *Neuroreport* 2002; **13**: 1631-1633

5 **Hantos MB**, Szalay F, Lakatos PL, Hegedus D, Firneisz G, Reiczigel J, Török T, Tekes K. Elevated plasma nociceptin level in patients with Wilson disease. *Brain Res Bull* 2002; **58**: 311-313

6 **Szalay F**, Hantos MB, Horvath A, Lakatos PL, Folhoffer A, Dunkel K, Hegedus D, Tekes K. Increased nociceptin/orphanin FQ plasma levels in hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 42-45

7 **Benvegnù L**, Gios M, Boccato S, Alberti A. Natural history of compensated viral cirrhosis: a prospective study on the incidence and hierarchy of major complications. *Gut* 2004; **53**: 744-749

8 **Daniele B**, Bencivenga A, Megna AS, Tinessa V. Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S108-S112

9 **Aoyagi Y**, Oguro M, Yanagi M, Mita Y, Suda T, Suzuki Y, Hata K, Ichii K, Asakura H. Clinical significance of simultaneous determinations of alpha-fetoprotein and des-gamma-carboxy prothrombin in monitoring recurrence in patients with hepatocellular carcinoma. *Cancer* 1996; **77**: 1781-1786

10 **Yamagamim H**, Moriyama M, Matsumura H, Aoki H, Shimizu T, Saito T, Kaneko M, Shioda A, Tanaka N, Arakawa Y. Serum concentrations of human hepatocyte growth factor is a useful indicator for predicting the occurrence of hepatocellular

carcinomas in C-viral chronic liver diseases. *Cancer* 2002; **95**: 824-834
11 **Spadaro A**, Ajello A, Morace C, Zirilli A, D'arrigo G, Luigiano C, Martino F, Bene A, Migliorato D, Turiano S, Ferraù O, Freni MA. Serum chromogranin-A in hepatocellular carcinoma: diagnostic utility and limits. *World J Gastroenterol* 2005; **11**: 1987-1990
12 **Chen PY**, Popovich PM. Correlation: Parametric and nonparametric measures. Thousand Oaks, CA: Sage Publications, 2002
13 **Pesarin F**. Multivariate permutation tests: with application in biostatistic. Chichester, New York, Weinheim, Brisbane, Singapore, Toronto: John Wiley & sons, LTD 2001
14 **Härdle W**, Simar L. Applied Multivariate Statistical Analysis. Berlin, Heidelberg, New York, Hong Kong, London, Milan, Paris, Tokyo: Springer-Verlag, 2003
15 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108
16 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
17 **Kim CK**, Lim JH, Lee WJ. Detection of hepatocellular carcinomas and dysplastic nodules in cirrhotic liver: accuracy of ultrasonography in transplant patients. *J Ultrasound Med* 2001; **20**: 99-104
18 **McIntire KR**, Waldmann TA, Moertel CG, Go VL. Serum alpha-fetoprotein in patients with neoplasms of the gastrointestinal tract. *Cancer Res* 1975; **35**: 991-996
19 **Bayati N**, Silverman AL, Gordon SC. Serum alpha-fetoprotein levels and liver histology in patients with chronic hepatitis C. *Am J Gastroenterol* 1998; **93**: 2452-2456
20 **Nguyen MH**, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Racial differences in effectiveness of alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. *Hepatology* 2002; **36**: 410-417
21 **Wang JB**, Johnson PS, Imai Y, Persico AM, Ozenberger BA, Eppler CM, Uhl GR. cDNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS Lett* 1994; **348**: 75-79
22 **Xie GX**, Ito E, Maruyama K, Suzuki Y, Sugano S, Sharma M, Pietruck C, Palmer PP. The promoter region of human prepronociceptin gene and its regulation by cyclic AMP and steroid hormones. *Gene* 1999; **238**: 427-436
23 **Horvath A**, Folhoffer A, Lakatos PL, Halosz J, Illyes G, Schaff Z, Hantos MB, Tekes K, Szalay F. Rising plasma nociceptin level during development of HCC: a case report. *World J Gastroenterol* 2004; **10**: 152-154
24 **Horvath A**, Folhoffer A, Csak T, Lakatos PL, Zalatnai A, Hantos MB, Tekes K, Szalay F. Increased nociceptin and nocistatin in plasma, cerebrospinal fluid and tumor tissue indicates altered opioid system in rats with experimental hepatocellular carcinoma. *J Hepatol* 2004; **40** S1: 79 AB 250
25 **Fiset ME**, Gilbert C, Poubelle PE, Pouliot M. Human neutrophils as a source of nociceptin: a novel link between pain and inflammation. *Biochemistry* 2003; **42**: 10498-10505
26 **Serhan CN**, Fierro IM, Chiang N, Pouliot M. Cutting edge: nociceptin stimulates neutrophil chemotaxis and recruitment: inhibition by aspirin-triggered-15-epi-lipoxin A4. *J Immunol* 2001; **166**: 3650-3654
27 **Schlicker E**, Morari M. Nociceptin/orphanin FQ and neurotransmitter release in the central nervous system. *Peptides* 2000; **21**: 1023-1029
28 **Helyes Z**, Németh J, Pintér E, Szolcsányi J. Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals. *Br J Pharmacol* 1997; **121**: 613-615

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RAPID COMMUNICATION

CCR5Δ32 mutation does not influence the susceptibility to HCV infection, severity of liver disease and response to therapy in patients with chronic hepatitis C

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Abstract

AIM: To study whether CCR5Δ32 mutation was associated with viral infection and severity of liver disease.

METHODS: Two hundred and fifty two histologically proven, chronic HCV patients (mean age: 41 ± 14 years; M/F: 164/88) were genotyped. PCR based genotyping of 32 bp deletion at the CCR5 locus was done. Four-hundred and eight matched healthy controls were studied to assess susceptibility to HCV infection. To assess correlation of immune gene polymorphism with severity of HCV related liver disease, patients with chronic HCV infection were divided into those with a fibrosis score of ⩽ 2 (mild) or > 2 (severe) and histological activity index (HAI) of ⩽ 5 or > 5. For correlation between CCR5Δ32 mutations and response to therapy, 129 patients who completed therapy were evaluated.

RESULTS: The majority (89.4%) of the patients were infected with genotype 3. The frequency of homozygous CCR5Δ32 mutants was comparable to HCV patients as compared to the healthy controls (0.7% *vs* 0%, P = 0.1). Further more, the frequency of CCR5Δ32 mutation was comparable in patients with mild or severe liver disease. (P = NS). There was also no association observed with response to therapy and CCR5Δ32 mutation.

CONCLUSION: CCR5Δ32 mutation does not have a role in disease susceptibility, severity or response to therapy in patients with chronic hepatitis C infection.

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Key words: β-chemokine receptor 32 bp deletion; CC chemokine ligand; Human leukocyte antigen; Histological activity index

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INTRODUCTION

HCV is the only known positive stranded RNA virus that causes persistent long-life infection in humans. Hepatitis C virus is an important cause for chronic liver disease and hepatocellular carcinoma in a proportion of patients with persistent infection. The mechanisms of chronicity and progression of liver disease are probably multifactorial and involve a rather complex interplay between the virus and the host. There has been a surge of interest in studying various immunomodulatory genes to answer these questions.

Hepatitis C virus being a hepatotropic virus requires the recruitment of virus specific T cells to the liver for viral clearance. Chemokines and interaction with their receptors may regulate the selective recruitment of primed Th-1 cells to the site of inflammation. It has been proposed that the immune response in chronic hepatitis C is compartmentalized, with a predominantly Th-2 or Th-0 response in the periphery and a Th-1 response in the liver[1-5]. In contrast, patients who clear the virus have a predominant peripheral Th-1 response. Moreover, differences in chemokine receptor expression between Th-1 and Th-2 cells might explain their selective recruitment to tissue. In particular, lymphocytes infiltrating HCV infected liver express high levels of the chemokine receptor (CCR5) and CXC

chemokine receptor (CXCR3). The CC chemokine macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) are important in immune surveillance. These chemokines bind to their corresponding receptors CCR1 and CCR5. Both these receptors are preferentially expressed on lymphocytes with a Th-1 cytokine secretion pattern. Chronic hepatitis C virus infection has been shown to lead to reduced surface expression of CCR1 and CCR5 on peripheral blood T lymphocytes[6] and more so, on CD8 lymphocytes. While the mechanism of altered chemokine receptor expression and chemokine responsiveness is still not clear, reduced expression would probably lead to a decrease in T lymphocyte migration in response to MIP-1α, MIP-1β and RANTES in chemotaxis assays.

The Δ32 mutation of the CCR5 gene was reported to be associated with inflammatory bowel disease by some but not all authors[7]. Furthermore, a negative correlation with susceptibility to rheumatoid arthritis has been described[8,9]. In contrast to these weak or contradicting correlations, the Δ32 mutation was found to be of paramount importance for protection against HIV infection. The β-chemokine receptor CCR5 appears to be the major co-receptor for entry of macrophage-tropic and non-syncitium-inducing (NSI) variants of HIV[10]. A 32 bp deletion mutation (Δ32) of the CCR5 gene encodes a non-functional protein. Homozygosity for the Δ32 deletion makes the CD4 T cells resistant to infection from NSI virus strains or delay HIV-1 disease progression in CCR5 Δ32 heterozygotes.

There is a controversy related to the role of CCR5Δ32 mutation in susceptibility to HCV infection and disease progression. Woitas *et al* have suggested that a genetically determined loss of CCR5 gene expression is linked to chronic HCV infection and high viral load[11]. On the other hand, a recent study by Promrat *et al* has shown that reduced expression of CCR5 and RANTES may lead to reduced hepatic inflammation and 59029 -G/A to improved response to interferon therapy in chronic hepatitis C[12]. Hellier *et al* also suggested a possible role of CCR5Δ32 polymorphism in the outcome of HCV infection[13].

The present case-control association study was undertaken to identify whether CCR5Δ32 mutation is associated with HCV disease progression and severity. We also compared the allele frequencies defined by CCR5Δ32 mutation in HCV patients with a control group of healthy individuals in order to identify a possible association with susceptibility to disease.

MATERIALS AND METHODS

Patients

Two hundred and fifty two patients with histologically proven chronic HCV infection were studied. The *inclusion criteria* were evidence of chronic hepatitis on liver biopsy and HCV RNA positivity on two occasions at baseline. The HCV RNA was detected as described elsewhere[14]. HCV genotyping was performed with the reverse hybridization line probe assay (LIPA; Innogenetics, Ghent, Belgium). Patients were *excluded* if they had hepatitis B virus (HBV) or HIV infection, history of heavy alcohol consumption (> 80 g/d for > 5 years), positivity for antinuclear or anti smooth muscle antibody (in 1:80 dilution), autoimmune liver disease, thyroid disease, diabetes mellitus or malaria. The Institutional Ethical Committee approved the study protocol. An informed consent was obtained for enrolling the patients. The clinical and biochemical assessment of the patients was done according to the study protocol. The histological examination of liver biopsies was done according to the modified Knodell scoring system[15].

Patients received either 3 million IU of IFN α 2b daily for 24 to 48 wk. A group of patients received pegylated IFN-alpha 2b (1 to 1.5 μg per wk) for either 24 or 48 wk depending on viral genotype. All patients received ribavirin given orally (patients with < 65 kg received 800 mg/d and 65-85 kg received 1000 mg/d). Outcome of treatment was classified as follows: End Therapy Response (ETR): patients with undetectable HCV RNA at the end of treatment; Sustained Virological Response (SVR): patients with undetectable HCV RNA 6 mo after the end-of-treatment; Non-Response (NR): failure to achieve viral clearance at the end-of-treatment or 6 mo there after.

Controls

Unrelated healthy adult subjects, with no previous history of liver disease and negative for HBV and HCV infection were included as controls. A total of 408 healthy subjects were included as controls. Both patients and control groups included Hindus, Muslims, and Christians. Patients and controls were prospectively matched for ethnic group.

DNA extraction and PCR- based genotyping of allelic variants

Genomic DNA was extracted from whole blood using a commercially available kit. The CCR5Δ32 deletion mutation was detected by PCR based techniques as mentioned elsewhere[16]. Primers amplified 2 fragments of 200 bp and 172 bp corresponding to wt and deleted CCR5 alleles. Primer sequences were CCR5L-5' TTA AAA GCC AGG ACG GTC AC 3' CCR5R-5' GAC CAG CCC CAA GAT GAC TA 3'. Cycling conditions were 96℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s. A final extension step of 72℃ for 10 min was applied. Amplified fragments for the CCR5 locus were resolved in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA sequencing

The CCR5Δ32 mutation was confirmed by sequencing. For DNA sequencing, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) was used.

Correlation of immune gene polymorphism and persistence of HCV infection

To study whether persistence of HCV infection is linked to immune gene polymorphism, patients with chronic HCV infection were compared with healthy controls.

Correlation of immune gene polymorphism and severity of HCV related liver disease

To study whether immune gene polymorphisms influence the course of HCV related liver disease, patients were

Table 1 Demographic profile of patients with chronic HCV infection and healthy controls

	Patients (*n* = 252)	Patients (Only genotype 3) (*n* = 187)	Healthy controls (*n* = 408)
Age (yr)			
Mean ± SD	40 ± 14	40 ± 14	33 ± 7
Male: Female	164:88	118:69	297:111
ALT (IU/L)			
Mean	112.3 ± 68.4	116 ± 70.1	29 ± 2
Range	(32-330)	(32-330)	
S. Albumin (g/L)	36 ± 4	34 ± 5	40 ± 3
HCV genotype (*n* = 209)			
I	17 (8.1%)		-
II	1 (0.5%)		
III	187 (89.4%)		-
IV	4 (1.5%)		-
Liver Biopsy			
Fibrosis score	2 ± 1	2 ± 1.2	-
HAI	5.5 ± 2.4	5.6 ± 2.5	-
Mode of Acquisition of HCV			
Blood transfusion	86		-
Needle stick injury	54		-
Intravenous drug use	10		-
Medical procedure	45		-
Unclear	57		

categorized on the basis of the stage of ALT levels (< 60 or ⩾ 60 IU/L), hepatic fibrosis and histological activity index (HAI). Patients were divided into those with a fibrosis score of ⩽ 2 (mild) or >2 (severe) and with a necroinflammatory score of ⩽ 5 (mild) or > 5 (severe).

Statistical analysis

The frequencies of CCR5Δ32 mutant allele were compared between patients with chronic HCV infection and controls by Chi-square test. Categorical variables were analyzed with χ^2 or Fisher's exact test. Two-sample *t*-tests were used to compare means for continuous variables and for non-normally distributed continuous variables, non-parametric test, Wilcoxon Mann-Whitney-U test was used for comparison of median values. Univariate analysis was used to assess associations between the various allelic variants and severity of liver disease.

Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies by χ^2-test. The distribution of genotypes between the patients and healthy controls were compared by contingency table analysis.

For all tests, a 2-tailed $P < 0.05$ was considered significant. The analysis was performed by using statistical software SPSS 12.0 software (SPSS, Chicago, IL).

RESULTS

Demographic profile

Two hundred and fifty two patients with chronic hepatitis C were studied. The baseline characteristics of the cases and controls in the study group are summarized in Table 1. The most common source of HCV infection was blood transfusion.

Table 2 Allele frequency of CCR5 variants in chronic HCV patients and healthy controls

CCR5	HCV Patients (*n* = 252)	Healthy Controls (*n* = 408)	*P* value
Wt/wt	247 (98%)	405 (99.3%)	0.07
Δ32mt carrier	5 (1.9%)	3 (0.7%)	0.3
Wt/Δ32	3 (1.2%)	3 (0.7%)	0.2
[a]Δ32mt/Δ32mt	2 (0.7%)	-	0.1

[a]The patients were genotype 1.

Viral genotype

HCV genotypes could be determined in 209 of the 252 (82.9%) patients. The predominant viral genotype was genotype 3a/3b. One hundred eighty seven (89.4%) patients had genotype 3, 17 (8.1%) had genotype 1, one patient had genotype 2 and 4 patients had genotype 4 (Table 1). No significant difference was observed in immune gene polymorphism and patients with different viral genotypes.

Distribution of CCR5 allelic variants

The frequency of CCR5Δ32 mutations in HCV patients and controls is shown in Table 2. Two hundred and forty seven (96.6%) HCV patients and 405 (99.3%) healthy controls had CCR5 wt/wt homozygosity. 3 (1.2%) of 252 patients had heterozygous and 2 (0.7%) had homozygous Δ32 mutations; overall 5 (1.9%) of the HCV patients had Δ32 mutant alleles. On the other hand, none of the healthy controls had a homozygous Δ32mt/Δ32mt pattern. Three healthy controls exhibited heterozygosity (Table 2). The difference between the patients and the controls was comparable but not significant. CCR5Δ32/Δ32 was present in HCV genotype 1 patients. Patients carrying CCR5Δ32 (CCR5Δ32 homozygote and heterozygote) mutation had no significant difference in the serum ALT level, degree of hepatic inflammation, fibrosis score and viral genotype compared with those patients who had wild type CCR5. All the allelic variants were in Hardy-Weinberg equilibrium.

Relation to serum alanine aminotranferase

We also used the mean serum ALT concentrations as an indicator of the activity of liver disease. However, no significant difference was observed for any of the genetic markers in patients with normal or without raised ALT (data not shown).

Relation to liver histology: Fibrosis and Histological inflammation

When patients were categorized based on the severity of hepatic fibrosis, 162 patients had mild and 90 patients had severe hepatic fibrosis. The frequency of CCR5Δ32 mutations was comparable in patients with mild or severe liver disease (P = NS) and it did not correlate with the severity of the liver disease (Table 3). Similarly CCR5Δ32 mutations did not correlate with histological severity

Response to therapy

Table 4 depicts the response pattern of the patients

Table 3 Correlation of Immune Gene Polymorphism and histological severity of HCV Related Liver Disease in Univariate analysis

Liver histology	CCR5-Genotype			*P*
	wt/wt	wt/Δ32	Δ32/Δ32	
Fibrosis				
⩽ 2 (*n* = 162)	154 (98%)	1 (0.6%)	2 (1.2%)	
> 2 (*n* = 90)	88 (97.8%)	2 (1.2%)	0	NS
HAI				
⩽ 5 (*n* = 162)	170 (98.3%)	1 (0.6%)	2 (1.2%)	
> 5 (*n* = 90)	77 (97.5%)	2 (2.5&)	0	NS

Table 4 Response patterns in HCV patients genotyped for CCR5

Response		CCR5-Genotype			*P*
		wt/wt	wt/Δ32	Δ32/Δ32	
IFN+ Riba/	Responders	86	1	-	NS
Peg IFN+ Rib	Non-responders	34	1	-	NS
	Relapsers	7	-	-	NS

enrolled in the study. When patients were categorized on the basis of the response pattern, no difference was observed in the distribution of alleles with respect to response rate. Of 252 patients, 129 patients received antiviral therapy. Eighty six patients had attained sustained virological response, 34 patients were non-responders while 7 patients relapsed after discontinuation of therapy and one patient developed decompensation and discontinued the therapy. Two patients who were CCR5Δ32/Δ32, did not receive therapy. There was also no difference in the response pattern among the heterozygotes.

DISCUSSION

Host genetic factors encoding for gene products, which are likely to be involved in the immune response following HCV infection, are likely to influence the disease susceptibility and progression. It has been demonstrated by studies which correlated polymorphisms or mutations of genes encoding for HLA subtypes[17-19], tumor necrosis factor[20-22], interleukin 10[23] or chemokine receptor 5 (CCR5) with disease susceptibility or treatment outcome in HCV.

In our population 1.9% (5/252) HCV patients were carriers of the CCR5Δ32 mutation as compared to 0.7% (3/408) of the general population. This refers to both homozygous and heterozygous mutations combined. Since the frequency of these mutations is rather low it is difficult to make definite statistical analysis between various groups. The reason for the variance in our results compared to the earlier studies is not easy to explain. This could probably be because the frequency of these mutations is rather low, thus it is difficult to make definite statistical analysis between various groups. An earlier study from India has reported a single heterozygote for CCR5Δ32 mutants[24]. However, the group had only genotyped 150 normal healthy individuals for the presence of Δ32 allele. We had adequate number of controls (*n* = 408). Woitas *et al* had observed a higher frequency of CCR5Δ32 homozygotes in chronic HCV patients[11]. In the present study, we observed a 0.7% occurrence of CCR5Δ32 mutants in chronic HCV patients but none in healthy controls, however the difference was not significant.

Apart from the sample size, a plausible explanation for these divergent results might be the differences in comorbidity of patients in the studies by Woitas and Nguyen[11,25], who had a substantial proportion of patients with haemophilia and/or concomitant hepatitis B. In the present study patients with haemophilia, hepatitis B or HIV were carefully excluded. Moreover, we did not detect elevated aminotransferase levels in carriers of the Δ32 mutation, which might also be due to the small number of homozygous carriers of the Δ32 mutants in the present study[26].

CCR5Δ32 mutants did not increase the likelihood of a more severe liver disease in our patients due to HCV infection (Table 3). Table 5 gives an overview of the published data in chronic HCV patients genotyped for CCR5Δ32 mutation. As shown in Table 5, the correlation of CCR5 Δ32 mutations with histological severity is controversial. Significant associations were found between CCR5Δ32 and reduced portal inflammation and milder fibrosis[13]. Liver inflammatory activity was found to be significantly reduced in Jewish Israeli patients infected with the hepatitis C virus carrying the CCR5Δ32 allele[32].Heterozygosity for CCR5Δ32 has been shown to be significantly associated with lower hepatic inflammatory scores[26]. However, other studies failed to find an association of CCR5Δ32 mutations with the histological severity.

Table 5 also shows distribution of viral genotypes in the published data for CCR5Δ32 mutation. It is known that the distribution of genotypes could be dependent on geographical distribution and possibly susceptibility. In all the previous studies, the associations have been studied primarily in the population of HCV patients infected with genotype 1. It might be possible that this could influence the susceptibility and severity of liver disease and could have led to variations in the results. The present study is the first study which has looked into the role of CCR5Δ32 mutation in genotype 3.

In the study population, nearly 161 of 182 (88.4%) patients in whom genotyping could be done, had genotype 3 infection. Most studies from the Indian subcontinent have reported similar frequency of genotype 3[34]. HCV 3a is a hepatitis virus strain that responds better to interferon IFN-α therapy than other HCV strains. IFN-α induces the production of CCL3, a CCR5 ligand, in the liver. It is thus possible that CCR5 is involved in a cascade of events or recruitment of immune cells, which negatively regulate the production of IFN-α in the liver. Loss of CCR5 expression due to CCR5Δ32 mutation could affect the course of hepatitis C, probably by interfering with cellular immune response. It has been suggested that CCR5Δ32/Δ32 mutant has no expression of CCR5 on the cell surface and henceforth is an ineffective HCV-specific immune response resulting in an immune imbalance to Th-1→Th-2 response[27].

It is therefore important to study the role of CCR5Δ32 mutation with response to therapy. Ahlenstiel *et al* have shown that response rates to interferon-alpha monotherapy are reduced in hepatitis C virus infected patients

Table 5 Analysis of published data in chronic HCV patients genotyped for CCR5Δ32 mutation

Authors	Susceptibility	Histological Severity	Response to therapy	Genotype	Patients studied (*n*)
Ahlensteil G *et al*[27,28]	Yes	Not mentioned	Lower ETR[1]	Genotype 1	59
				Genotype 2	3
				Genotype 3	9
				Genotype 4	3
				Undetermined	4
Glas J *et al*[29]	No	Not mentioned	No	Genotype 1/4	37
				Genotype 2/3	25
Goulding C *et al*[26]	[2]Spontaneous viral clearance	[2]Lower hepatic inflammation	Not mentioned	Genotype 1b	283
Hellier *et al*[13]	No	Decreased portal inflammation	No	Stratification not mentioned	
Konishi *et al*[30]	No	No	No	Serotype 1	53
				Serotype 2	48
				Serotype 1 + 2	4
Mascheretti *et al*[31]	No	No	No	Genotype 1	358
				Genotype 2	14
				Genotype 3	79
				Genotype 4	14
Promrat *et al*[12]	No	No	No	Genotype 1	243
				Genotype 2	34
				Genotype 3	16
				Genotype 4	3
				Genotype 5	1
				Undetermined	42
Wald *et al*[32]	No	Significantly reduced	Not mentioned	Genotype 1	5
				Genotype 2	2
				Genotype 3	9
				Undetermined	31
Wasmuth *et al*[33]	No	No	No	Genotype 1	213
				Non-Genotype 1	117
Woitas *et al*[11]	Yes	Not mentioned	Not mentioned	Genotype 1	95
				Genotype 2	16
				Genotype 3	10
				Genotype 4	6
				Multiple genotype	5
				Undetermined	2
Present study Goyal *et al*	No	No	No	Genotype 1	16
				Genotype 2	1
				Genotype 3	161
				Genotype 4	4
				Undetermined	70

[1] All analysis mentioned w.r.t the CCR5Δ32 carriers. [2] Heterozygotes for CCR5Δ32 mutation.

carrying the CCR5Δ32 mutation[28]. It is difficult to comment on the role of CCR5 mutations in HCV infection with genotype 3 in our population, since this is the most common genotype. Also, we had 0.7% allelic frequency of CCR5Δ32 and since 98% of HCV patients had CCR5 wt/wt, speculating a higher SVR in 3a genotype is not possible. Our patients had received combination therapy of interferon/pegylated interferon and ribavirin. However it has been suggested that the interferon and ribavirin combination treatment may overcome this negative effect of CCR5Δ32[28]. Thus, in population a infected with genotype 3 HCV, CCR5Δ32 mutations do not influence the response to combination therapy with interferon and ribavirin.

In conclusion, our results indicate that CCR5Δ32 mutation does not influence the susceptibility and severity of liver disease in chronic hepatitis C patients. Moreover, CCR5Δ32 mutation does not influence the response to therapy.

REFERENCES

1 **Diepolder HM**, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 1995; **346**: 1006-1007

2 **Dumoulin FL**, Bach A, Leifeld L, El-Bakri M, Fischer HP, Sauerbruch T, Spengler U. Semiquantitative analysis of intrahepatic cytokine mRNAs in chronic hepatitis C. *J Infect Dis* 1997; **175**: 681-685

3 **Napoli J**, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *Hepatology* 1996; **24**: 759-765

4 **Tsai SL**, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* 1997; **25**: 449-458

5 **Woitas RP**, Lechmann M, Jung G, Kaiser R, Sauerbruch T, Spengler U. CD30 induction and cytokine profiles in hepatitis C virus core-specific peripheral blood T lymphocytes. *J Immu-*

nol 1997; **159**: 1012-1018
6 **Lichterfeld M**, Leifeld L, Nischalke HD, Rockstroh JK, Hess L, Sauerbruch T, Spengler U. Reduced CC chemokine receptor (CCR) 1 and CCR5 surface expression on peripheral blood T lymphocytes from patients with chronic hepatitis C infection. *J Infect Dis* 2002; **185**: 1803-1807
7 **Martin K**, Heinzlmann M, Borchers R, Mack M, Loeschke K, Folwaczny C. Delta 32 mutation of the chemokine-receptor 5 gene in inflammatory bowel disease. *Clin Immunol* 2001; **98**: 18-22
8 **Garred P**, Madsen HO, Petersen J, Marquart H, Hansen TM, Freiesleben Sørensen S, Volck B, Svejgaard A, Andersen V. CC chemokine receptor 5 polymorphism in rheumatoid arthritis. *J Rheumatol* 1998; **25**: 1462-1465
9 **Gómez-Reino JJ**, Pablos JL, Carreira PE, Santiago B, Serrano L, Vicario JL, Balsa A, Figueroa M, de Juan MD. Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. *Arthritis Rheum* 1999; **42**: 989-992
10 **Dragic T**, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996; **381**: 667-673
11 **Woitas RP**, Ahlenstiel G, Iwan A, Rockstroh JK, Brackmann HH, Kupfer B, Matz B, Offergeld R, Sauerbruch T, Spengler U. Frequency of the HIV-protective CC chemokine receptor 5-Delta32/Delta32 genotype is increased in hepatitis C. *Gastroenterology* 2002; **122**: 1721-1728
12 **Promrat K**, McDermott DH, Gonzalez CM, Kleiner DE, Koziol DE, Lessie M, Merrell M, Soza A, Heller T, Ghany M, Park Y, Alter HJ, Hoofnagle JH, Murphy PM, Liang TJ. Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* 2003; **124**: 352-360
13 **Hellier S**, Frodsham AJ, Hennig BJ, Klenerman P, Knapp S, Ramaley P, Satsangi J, Wright M, Zhang L, Thomas HC, Thursz M, Hill AV. Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. *Hepatology* 2003; **38**: 1468-1476
14 **Cha TA**, Kolberg J, Irvine B, Stempien M, Beall E, Yano M, Choo QL, Houghton M, Kuo G, Han JH. Use of a signature nucleotide sequence of hepatitis C virus for detection of viral RNA in human serum and plasma. *J Clin Microbiol* 1991; **29**: 2528-2534
15 **Goodman ZD**, Ishak KG. Histopathology of hepatitis C virus infection. *Semin Liver Dis* 1995; **15**: 70-81
16 **Barber Y**, Rubio C, Fernández E, Rubio M, Fibla J. Host genetic background at CCR5 chemokine receptor and vitamin D receptor loci and human immunodeficiency virus (HIV) type 1 disease progression among HIV-seropositive injection drug users. *J Infect Dis* 2001; **184**: 1279-1288
17 **Bosi I**, Ancora G, Mantovani W, Miniero R, Verucchi G, Attard L, Venturi V, Papa I, Sandri F, Dallacasa P, Salvioli GP. HLA DR13 and HCV vertical infection. *Pediatr Res* 2002; **51**: 746-749
18 **Vejbaesya S**, Songsivilai S, Tanwandee T, Rachaibun S, Chantangpol R, Dharakul T. HLA association with hepatitis C virus infection. *Hum Immunol* 2000; **61**: 348-353
19 **Zavaglia C**, Martinetti M, Silini E, Bottelli R, Daielli C, Asti M, Airoldi A, Salvaneschi L, Mondelli MU, Ideo G. Association between HLA class II alleles and protection from or susceptibility to chronic hepatitis C. *J Hepatol* 1998; **28**: 1-7
20 **Goyal A**, Kazim SN, Sakhuja P, Malhotra V, Arora N, Sarin SK. Association of TNF-beta polymorphism with disease severity among patients infected with hepatitis C virus. *J Med Virol* 2004; **72**: 60-65
21 **Höhler T,** Kruger A, Gerken G, Schneider PM, Meyer zum Büschenfelde KH, Rittner C. Tumor necrosis factor alpha promoter polymorphism at position -238 is associated with chronic active hepatitis C infection. *J Med Virol* 1998; **54**: 173-177
22 **Rosen HR**, McHutchison JG, Conrad AJ, Lentz JJ, Marousek G, Rose SL, Zaman A, Taylor K, Chou S. Tumor necrosis factor genetic polymorphisms and response to antiviral therapy in patients with chronic hepatitis C. *Am J Gastroenterol* 2002; **97**: 714-720
23 **Yee LJ**, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; **33**: 708-712
24 **Husain S**, Goila R, Shahi S, Banerjea A. First report of a healthy Indian heterozygous for delta 32 mutant of HIV-1 co-receptor-CCR5 gene. *Gene* 1998; **207**: 141-147
25 **Nguyêñ GT**, Carrington M, Beeler JA, Dean M, Aledort LM, Blatt PM, Cohen AR, DiMichele D, Eyster ME, Kessler CM, Konkle B, Leissinger C, Luban N, O'Brien SJ, Goedert JJ, O' Brien TR. Phenotypic expressions of CCR5-delta32/delta32 homozygosity. *J Acquir Immune Defic Syndr* 1999; **22**: 75-82
26 **Goulding C**, McManus R, Murphy A, MacDonald G, Barrett S, Crowe J, Hegarty J, McKiernan S, Kelleher D. The CCR5-delta32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source. *Gut* 2005; **54**: 1157-1161
27 **Ahlenstiel G**, Woitas RP, Rockstroh J, Spengler U. CC-chemokine receptor 5 (CCR5) in hepatitis C--at the crossroads of the antiviral immune response? *J Antimicrob Chemother* 2004; **53**: 895-898
28 **Ahlenstiel G**, Berg T, Woitas RP, Grünhage F, Iwan A, Hess L, Brackmann HH, Kupfer B, Schernick A, Sauerbruch T, Spengler U. Effects of the CCR5-Delta32 mutation on antiviral treatment in chronic hepatitis C. *J Hepatol* 2003; **39**: 245-252
29 **Glas J**, Török HP, Simperl C, König A, Martin K, Schmidt F, Schaefer M, Schiemann U, Folwaczny C. The Delta 32 mutation of the chemokine-receptor 5 gene neither is correlated with chronic hepatitis C nor does it predict response to therapy with interferon-alpha and ribavirin. *Clin Immunol* 2003; **108**: 46-50
30 **Konishi I**, Horiike N, Hiasa Y, Michitaka K, Onji M. CCR5 promoter polymorphism influences the interferon response of patients with chronic hepatitis C in Japan. *Intervirology* 2004; **47**: 114-1120
31 **Mascheretti S**, Hinrichsen H, Ross S, Buggisch P, Hampe J, Foelsch UR, Schreiber S. Genetic variants in the CCR gene cluster and spontaneous viral elimination in hepatitis C-infected patients. *Clin Exp Immunol* 2004; **136**: 328-333
32 **Wald O**, Pappo O, Ari ZB, Azzaria E, Wiess ID, Gafnovitch I, Wald H, Spengler U, Galun E, Peled A. The CCR5Delta32 allele is associated with reduced liver inflammation in hepatitis C virus infection. *Eur J Immunogenet* 2004; **31**: 249-252
33 **Wasmuth HE**, Werth A, Mueller T, Berg T, Dietrich CG, Geier A, Schirin-Sokhan R, Gartung C, Lorenzen J, Matern S, Lammert F. CC chemokine receptor 5 delta32 polymorphism in two independent cohorts of hepatitis C virus infected patients without hemophilia. *J Mol Med* (Berl) 2004; **82**: 64-69
34 **Singh S**, Malhotra V, Sarin SK. Distribution of hepatitis C virus genotypes in patients with chronic hepatitis C infection in India. *Indian J Med Res* 2004; **119**: 145-148

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RAPID COMMUNICATION

Coimmunization with IL-15 plasmid enhances the longevity of CD8 T cells induced by DNA encoding hepatitis B virus core antigen

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Abstract

AIM: To test the feasibility of delivering a plasmid encoding IL-15 as a DNA vaccine adjuvant for improving the immune responses induced by hepatitis B virus core gene DNA vaccine.

METHODS: We used RT-PCR based strategies to develop IL-15 expression constructs. We first confirmed that the gene could be expressed in *Escherichia coli* due to the poor expression of IL-15. Then the bioactivity of IL-15 plasmid expression product was identified by CTLL-2 proliferation assay. One hundred micrograms of DNA from each of the IL-15 eukaryotic expressed plasmid and the recombinant plasmid harboring DNA encoding the 144 amino acids of the N-terminus of HBV core gene (abbreviated pHBc144) was used to co-immunize C57 BL/6 mice. The titer of anti-HBcIgG was detected by ELISA and the antigen-specific $CD8^+$ T cells ($CD8^+IFN\text{-}\gamma^+$ T cells) were detected by intracellular cytokine staining at different time points.

RESULTS: After co-immunization by pIL-15 and pHBc144 DNA vaccine the antigen-specific $CD8^+$ cells of mice increased gradually, the first peak of immune response appeared 14 d later, then the number of antigen-specific $CD8^+$ Ts cells decreased gradually and maintained at a steady level in 3 mo. After boosting, the number of antigen-specific $CD8^+$ T cells reached the second peak 10 d later with a double of the 1st peak, then the number of antigen-specific $CD8^+$ T cells decreased slowly. IL-15 as a gene adjuvant had no significant effect on humoral immune responses induced by hepatitis B virus core gene DNA vaccine, but increased the memory antigen-specific $CD8^+$ T cells induced by hepatitis B virus core gene DNA vaccine.

CONCLUSION: DNA vaccine constructed by HBc Ag 1-144 amino acid induces effective cell immunity, and cytokine plasmid-delivered IL-15 enhances the longevity of $CD8^+$ T cells.

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Key words: Vaccine; DNA vaccine; Hepatitis B virus core antigen

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INTRODUCTION

The final goal of a vaccine is to make body acquire long-term and effective protection. However the long-term and effective protection depends on the quality and quantity of memory T cells. IL-15 mRNA is constitutively expressed by various cells and tissues such as placenta, skeletal muscle, kidney, epithelial cells, synovial cells, and macrophages[1]. IL-15 is essential for $CD8^+$ memory T-cell generation and maintenance, and is involved in the maintenance of antigen-specific memory $CD8^+$ T cells. The number of memory-type (CD44highLy6C) $CD8^+$ T cells increases significantly in the periphery lymphoid tissue of IL-15 Tg mice, producing IL-15, while the number of antigen-specific memory $CD8^+$ T cells declines in IL-15$^-$ and IL-15R$^-$ deficient mice[2].

IL-15 has attracted great attention because of its important effect on memory $CD8^+$ T cells[3-5]. This report describes the IL-15 mRNA extraction from

human peripheral blood mononuclear cells (PBMCs) and the molecular cloning of IL-15. We demonstrated that recombinant IL-15 was expressed in *Escherichia coli* and showed bioactivity in COS cells. Our results suggest that IL-15 plasmid has effect on the memory phase of cellular immune response induced by HBc DNA vaccine, indicating that plasmid IL-15 functions as a candidate adjuvant and can be used in for vaccine or immunotherapeutic studies.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice at the age of 6-8 wk were purchased from Shanghai Sippr-Bk Company and allowed to acclimate for at least 2 wk prior to use. The mice were housed in laminar airflow cages with free access to food and water *ad libitum*. Analyses of specimens from those animals revealed no evidence of exposure to viral, bacterial or parasitic pathogens during these studies.

Plasmids and peptides

The HBcAg-expression plasmid pHBc144 was constructed by inserting a gene encoding hepatitis B core antigen N' end 144 amino acid into a vector (pcDNA3, Invitrogen), driven by the cytomegalovirus (CMV) immediate early promoter. The secreted HBcAg could be detected in the supernatant of cell culture transiently transfected with pHBc144[6]. Anti-HBc could be detected in the serum of Balb/c mice, which were injected with pHBc144 DNA plasmid.

The HBV core Ag peptides were synthesized and generously provided by Dr. Rafi Ahmed (Emory University, GA)

Cell culture

PBMCs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation over Ficoll. Fresh whole blood was drawn, heparinized and gently mixed with one volume of RPMI1640. The diluted blood was layered over Ficoll and centrifuged at 800 g, for 20 min at 4℃. PBMC were collected from the interface between the plasma and the density gradient solution. After being washed in PBS, PBMCs were resuspended in RPMI 1640 supplemented with 5 mg/mL LPS, 200 mmol/L L-guatmine, 10% FCS, 20 mmol/L Hepes, 100 U/mL penicillin, and 100 μg/mL streptomycin, at a final cell concentration of 5×10^6/mL. Then the PBMCs were cultured in 24-well plates for 6 h.

RT-PCR and cDNA cloning of Interlukin-15

RNA extraction as well as RT-PCR analysis were performed as previously described[7]. Briefly, total RNA was purified using Trizol (Invitrogen Life Technologies) following the manufacturer's instructions. A 4 μg aliquot of total cellular RNA was reverse transcribed using random hexanucleotide primers and the Superscript Ⅱ preamplification system (Invitrogen Life Technologies). One-tenth of the cDNA obtained was amplified in a 20-pl reaction, using 1 U of P*wo* DNA Polymerase Expand™ High Fidelity PCR System (Boehringer Mannheim) and the reagents provided with the preamplification system. Thirty-five cycles of PCR amplification were performed at 94℃ for 2 min, at 60℃ for 1 min and at 72℃ for 1 min; followed by a final extension at 72℃ for 7 min. Ten microliters of the reaction products was analyzed on 1% agarose gel. The primer sequences are sense: 5'-GCG*GAATTC*AT-GAGAATTTCGAAACCACATTTGAG-3'; antisense: 5'-GCG*CTCGAG*AATCAATTGCAATCAAGAAGT-GT-3'. The amplified cDNA was ultimately digested with *Eco*R I and *Xho* I and cloned into pcDNA3 and pET2c. The sequences of the IL-15 DNA insert were verified by sequencing with the dideoxy chain termination method (Bioasia Co., Shanghai). Sequences were analyzed by Vector NTI Suite software package (InfoMax).

Expression of recombinant protein

Escherichia coli strain *BL21*(*DE3*) (Novagen) was transformed by the pET2c-IL-15 plasmid. The detailed protocol for production of fusion proteins was as follows. *E.coli* cells were grown to absorbance 0.6 at 600 nm, then expression was induced with 0.4 mmol/L isopropyl-D-thiogalac-toside for 4 h at 37℃. The bacterial cells were harvested, the cell pellet was suspended in 50 mL MTPBS lysis buffer (150 mmol/L NaCl, 16 mmol/L NaH_2PO_4, 4 mmol/L Na_2HPO_4, pH 7.3, 1% TritonX-100) per liter of culture volume. The cells were sonicated three times, 10 s each time (Sinifer cell disrupter B-30; Branson Sonic Power), and centrifuged at 15 000 g for 10 min at 4℃. The supernatant was collected for SDS-PAGE and Western blotting.

Western blotting

E.coli cells were cultured for an indicated period of time, lysed by adding 2X Laemmli SDS sample buffer and boiled for 5 min in a water bath. The proteins were resolved by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immun-blot PVDF; Bio-Rad). Nonspecific binding sites were blocked by incubating the blot in blocking buffer (5% nonfat dry milk in wash buffer) for 1 h at room temperature. The blot was washed three times (5 min each time) with wash buffer (0.5% Tween 20, 200 mmol/L NaCl, and 50 mmol/L Tris, pH 7.5), and incubated with the appropriate primary Ab overnight at 4℃. The blot was then washed three times (5 min each time) and incubated with the corresponding secondary Ab-HRP conjugate for 1 h at room temperature. The blot was washed three times again (5 min each time), and proteins were detected.

CTLL-2 proliferation assay

For CTLL-2 bioassays, COS cells were transfected with 30 μg of IL-15 expression constructs following a standard calcium-phosphate-mediated transfection protocol[7]. For these studies roughly $2\text{-}3 \times 10^5$ adherent COS cells/well were transfected in six-well 35-mm plates. After 24 h, the medium was replaced with fresh complete DMEM, supernatants were harvested 72 h after the transfection for assay.

Aliquots (100 μL) of the supernatant and serial two-

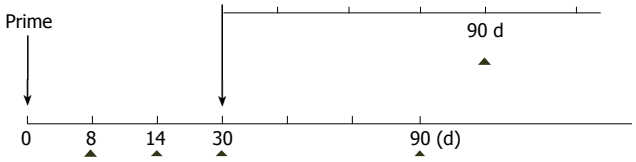


Figure 1 Immunization schedule of pHBc144 and pIL-15 plasmid.

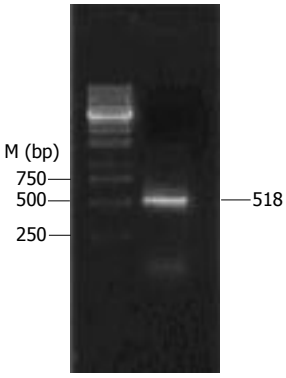


Figure 2 Electrophoretogram of PCR products. The human IL-15 coding sequence (GenBank U14407) was RT-PCR amplified from human PBL mRNA stimulated with LPS.

fold dilution were added to 96-well microtiter plates containing 1×10^5 CTLL-2 cells in a final volume of 200 μL. The cultures were incubated for 18 h at 37℃ and pulse-labeled for another 6 h with 0.5 μC*i* [^{3}H] thymidine. Thymidine incorporation was quantified by liquid scintillation spectrometry[8] (BD). A standard curve was plotted from proliferation induced by standard murine rIL-2.

Immunization and sample collection

Plasmids were amplified in *Escherichia coli* and plasmid DNA was purified by using anion exchange column (Endofree plasmid purification kit, Qiagen, Chatworth, Calif) according to the manufacturer's instructions. The plasmid DNA was dissolved in sterile physiological saline, and the final concentration was 1 μg/mL.

C57BL/6 (H-2^b) mice were vaccinated at the age of 6-8 wk by injection of DNA in 0.9% saline (w/v), into the quadriceps of each hind limb as previously described[9]. Injection volume was 50 μL per leg and total DNA dose was 50 μg per leg. On d 30, all mice were booster-immunized with the same DNA. On d 0, 8, 14, 30, 90 after priming and d 90 after boostering, blood samples were collected from each mouse by orbital bleeding (Figure 1). Serum was separated from each blood sample and used for measurement of the anti-HBcAb titer. On the other hand, mouse blood was obtained using heparin as an anticoagulant. The blood was diluted with RPMI 1640. PBMCs were obtained by density gradient centrifugation over HISTOPAQUE-1083 (Sigma), washed three times with RPMI 1640 and counted afterward. The spleens were removed for the measurement of cytokine production.

Detection of anti-HBcIgG and subclasses

Anti-HBc antibody was titrated with ELISA kit (Shanghai Infectious Diseases' Hospital). Each serum sample was made in a series of three-fold dilution. Goat anti-mouse IgG-HRP conjugate (The Binding Site Limited Co, England) was used as the secondary antibody and O-pheylenediamine (OPD) was used as a substrate. The plates were read with Titerek Multiskan MCC/340 at A_{450}.The wells with an absorbance of ≥ 0.1 (above background, 0.020) were scored as positive.

Intracellular cytokine staining and flow cytometric (FCM) analysis

For intracellular cytokine detection of IFN-γ, PBMCs and single suspension of splenocytes were plated in a 96-well plate (Costar) at a density of 1×10^6 cells/well in a final volume of 200 μL. Cells were stimulated with peptides for 5 h. Brefeldin A (final concentration 2 μg/mL) was added to the cultures during stimulation. At the end of the incubation, the cells were washed twice and stained for 30 min at 4℃ with phycoerythrin (PE)-conjugated anti-mouse CD8 (BD Bioscience) in FACS buffer [2% FCS, 0.1% sodium azide in phosphate buffered saline (PBS)]. Cells were collected, washed and incubated in permeabilization buffer for 20 min on ice. The permeabilized cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IFN-γ (BD PharMingen, San Diego, CA) for 30 min. Cells were washed, fixed with 2% (w/v) paraformaldehyde, and acquired with a FACSCalibur flow cytometer (Becton Dickinson, Boston, MA). Forward and side scatters were collected on a linear scale, while the PE and FITC signals were collected on a 4-decade log scale. Overlaps of emission spectra were electronically compensated. The debris were eliminated using the threshold on forward scatter and 100 000 events were acquired with CellQuest software (BD, Biosciences).

Statistical analysis

Statistical analyses of data were performed using SPSS 10.0 for windows (SPSS, Inc., Chicago, IL).

RESULTS

cDNA cloning

PBMCs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation over Ficoll. A total of 5×10^5 PBMCs were transferred into 24-well plates and cultured for 6 h with 5 μg/mL LPS. RNA extraction and RT-PCR were performed (Figure 2). The complete human IL-15 cDNA (GenBank accession No. U14407) was 518 bp long and was comprised of 146 bp signal peptide (IL-15sp) and a IL-15 mature peptide gene sequence (IL-15mp).

Amplified cDNA was ultimately digested with *Eco*R I and *Xho* I and cloned into pcDNA3. The sequences of IL-15 DNA insert were confirmed and this plasmid was named pIL-15.

For generation of the IL-15 prokaryotic expression construct, the IL-15 mp coding sequence was PCR amplified from pIL-15. The resulting fragments were digested with *Eco*R I and *Xho* I and cloned into pET2c named pET-IL-15 (data not shown) .

Expression of IL-15 in Escherichia coli

Escherichia coli strain *BL21*(*DE3*) (Novagen) was transformed by the pET-IL-15 plasmid. After induction by

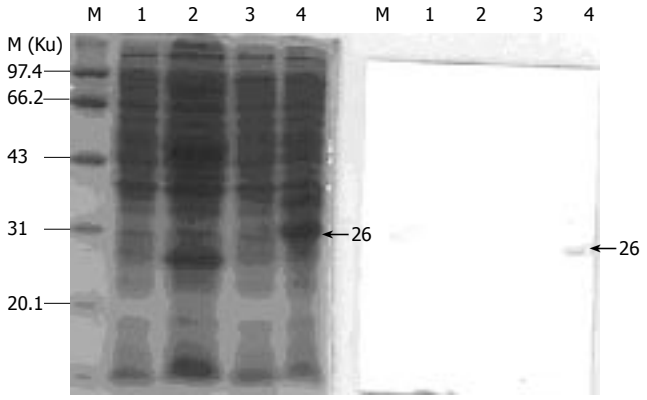


Figure 3 SDS-PAGE and Western blotting of recombinant polypeptides. M: polypeptide molecular marker; lane 1: BL21; lane 2: pET32c ; lane 3: pET-15 uninduced ; lane 4: pET-15 induced.

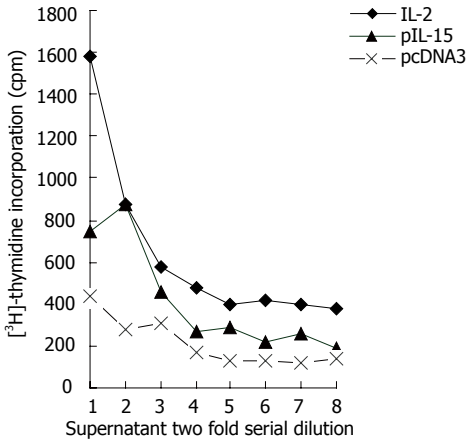


Figure 4 IL-15 bioactivity in supernatants of cells transfected with pIL-15 expression plasmid. COS cells were transfected with pIL-15 (▲) expression constructs or vector pcDNA3 (×), and the supernatants were collected 72 h after transfection. The supernatants were tested by CTLL-2 bioassay to measure IL-15 bioactivity. IL-2 (5×10^3 U/ mL) was used as a positive control.

IPTG and temperature, the supernatant of bacteria lysates was taken for SDS-PAGE and Western blotting. IL-15 fragments could be expressed in pET-32c vector and its molecular weight was M_r 26 000 (Figure 3).

Production of IL-15 by COS cells

The IL-15 was cloned into the pcDNA3 vector with CMV promoter (Invitrogen) and transfected into COS cells. Forty-eight hours after transfection, the COS supernatants were collected and subjected to CTLL-2 proliferation assay. CTLL-2 cell ^{3}H-TdR cpm was related to the dilution fold of the COS supernatant. When the dilution fold of transfection supernatant increased, the incorporation of ^{3}H-TdR cpm decreased. The incorporation ^{3}H-TdR cpm of pIL-15-transfected COS supernatant was higher than that of vector-tansfected COS supernatant (Figure 4). So we could detect IL-15 activity in the supernatants of COS cells transfected with pIL-15.

Kinetics of antigen-specific CD8 responses following DNA vaccination and cytokine expression profiles of antigen-specific T cells

Both primary and memory antigen-experienced CD8$^+$ T cells rapidly upregulated cytokine production after encountering cells displaying their cognate peptide ligand in association with a major histocompatibility complex class I molecule. We used ICCS to identify cytokine-producing HBc-specific CD8$^+$ memory T cells directly *ex vivo* after DNA inoculation prior to this study, but it was unclear how quickly these responses developed when IL-15 as a gene adjuvant was coinjected with HBc DNA vaccine. Therefore, we used ICCS to directly enumerate antigen-specific effector CD8$^+$ T cells in the spleens of mice at various time points after DNA immunization (pHBc144 and/or pIL-15). The average percentage of peptide-specific CD8$^+$ T cells detected in three mice analyzed at each time point for each mode of immunization is shown in Figure 5A.

Seven days after-immunization, antigen specific CD8$^+$ T cells were present at a frequency of less than 0.1% in all of the vaccinated mice (both pHBc144 + pcDNA3 group and pHBc144 + pIL-15 groups), and therefore accurate analysis of IFN-γ production was not possible (Figure 5A). By d 14, 2.64% ± 1.07% of the HBcAg specific CD8$^+$ T cells primed by pHBc144 + pIL-15 DNA vaccination secreted IFN-γ, and at 30 d after-immunization, 0.39% ± 0.21% of the antigen-specific cells were positive. A similar transition was observed in CD8$^+$ T cells primed by pHBc144 + pcDNA3 DNA vaccination (2.26% ± 2.50% on d 14 and 0.42% ± 0.06% on d 30). Thus, the cytokine expression patterns of HBcAg-specific T cells were similar regardless of the pIL-15 immunization.

These proportions decreased gradually, by d 90 post-priming, 0.48% ± 0.36% of the antigen-specific CD8$^+$ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN-γ, while 0.16% ± 0.04% of the antigen-specific CD8$^+$ T cells in pHBc144 and pcDNA3 co-immunized group were IFN-γ$^+$CD8$^+$ T cells (Figure 6).

After being boosted, the HBc Ag specific T cells expanded rapidly, and by d 90 post-boost the specific memory cells could still be detected, sustaining a higher level than that for the first response. On d 90 after boosting, 0.28% ± 0.04% of the antigen-specific CD8$^+$ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN-γ, while 0.11% ± 0.04% of the antigen-specific CD8$^+$ T cells in pHBc144 and pcDNA3 co-immunized group were IFN-γ$^+$ CD8$^+$ T cells (Figures 6A and B).

Kinetics of HBcAg specific CD8$^+$ T-cells in the circulation induced by DNA vaccine

For examining the kinetics and distribution of antigen specific CD8$^+$ T-cells in the non-lymphoid tissue induced by DNA vaccination, we separated the lymphocytes from blood. The kinetics of HBcAg-specific CD8$^+$ T cells in the circulation stimulated with pHBc144 showed a similar pattern with that of the spleen (Figure 5). However the number of HBcAg-specific CD8$^+$ T cells in PBMCs was lower than that in the spleen at the same time point. Eight days after DNA vaccination, HBcAg-specific CD8$^+$ T cells were detectable. By d 14 the number of IFN-γ$^+$ CD8$^+$ T cells in PBMCs reached the peak and then dropped gradu-

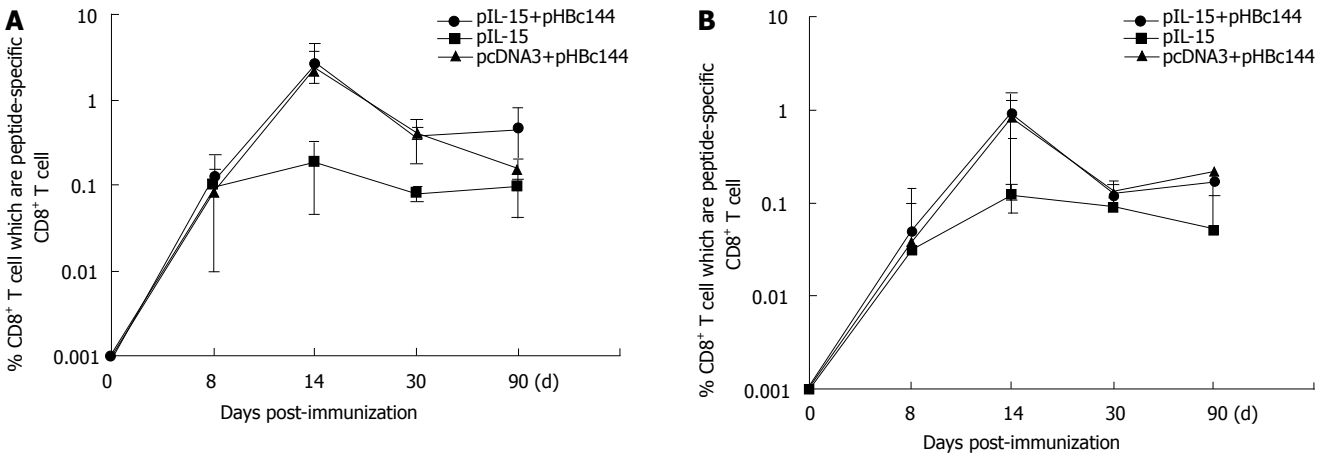


Figure 5 Kinetics of antigen-specific CD8$^+$ T-cell response in spleen (**A**) and PBMCs (**B**) following DNA immunization. Mice were immunized with pHBc144 plasmid DNA or vector DNA intramuscularly. At various time points, splenocytes and PBMCs were harvested, stimulated for 5 h with specific peptide, double stained with anti-CD8a-PE and anti-IFN-γ-APC. A total of 1 × 10^5 events were acquired by a flow cytometer. The indicated values on the lines of the figure (plotted on a log$_{10}$ scale) reflect the percentage of peptide-specific CD8$^+$ T-cells to total CD8$^+$ T-cells in the spleens or PBMCs (about three mice/group at each time point). The cut-off value was 0.1%.

Table 1 Total CD4$^+$ and CD8$^+$ T-cells detected in spleen and PBMC from mice co-immunized with pHBc144 and pIL-15 following DNA immunization

A Spleen

Group	d 8		d 14		d 30		d 90		booster d 90	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
pIL-15+pHBc144	13.35 ± 1.00	8.31 ± 2.08	17.89 ± 0.83	15.15 ± 0.88	22.02 ± 2.12	12.63 ± 0.51	20.23 ± 3.07	23.41 ± 0.65	11.76 ± 2.36	12.10 ± 0.59
pcDNA3+pHBc144	14.08 ± 1.32	8.24 ± 1.08	16.69 ± 2.21	14.75 ± 0.87	19.56 ± 1.23	14.58 ± 0.98	19.77 ± 3.61	22.37 ± 1.53	12.03 ± 2.50	13.43 ± 1.93
pIL-15	14.43	10.12	14.69 ± 2.25	10.31 ± 1.94	19.76 ± 1.41	13.38 ± 1.53	19.04 ± 1.75	15.72 ± 3.08	11.08 ± 0.21	12.76 ± 2.55

B PBMC

Group	d 8		d 14		d 30		d 90		booster d 90	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
pIL-15+pHBc144	13.05 ± 1.75	7.42 ± 1.41	15.93 ± 2.95	10.67 ± 1.87	19.10 ± 3.70	10.06 ± 0.47	16.94 ± 1.19	8.61 ± 1.20	8.66 ± 0.13	12.78 ± 1.76
pcDNA3+pHBc144	15.49 ± 4.77	8.35 ± 1.84	18.30 ± 3.56	12.24 ± 1.27	22.97 ±3.17	12.79 ± 4.39	7.50 ± 0.58	7.18 ± 2.93	10.09 ± 5.19	16.20 ± 1.48
pcDNA3+pHBc144	14.85	8.92	17.98 ± 3.20	7.68 ± 3.46	21.70 ± 5.44	12.51 ± 1.16	12.58 ± 0.66	9.43 ± 1.36	8.36 ± 3.53	10.34 ± 2.09

Mice were injected with 100 μg of pIL-15 or pHBc144 or both pIL-15 and pHBc144 plasmids intramuscularly. Single-cell suspensions were prepared. Cells were stained with anti-CD4-APC and anti-CD8-PE. The stained cells were analyzed with a FACScan. The data from each group at each time point represent the mean ± SD (n = 3). (A) the total CD4 and CD8 in the spleen. (B) the total CD4 and CD8 in the PBMC.

ally. These proportions decreased gradually until d 90 post-priming, 0.17% ± 0.05% of the antigen-specific CD8$^+$ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN-γ, while 0.22% ± 0.01% antigen-specific CD8$^+$ T cells in pHBc144 and pcDNA3 co-immunized group were IFN-γ$^+$ CD8$^+$ T cells.

On d 90, after boosting, 0.20% ± 0.13% of the antigen-specific CD8$^+$ T cells induced by pHBc144 and pIL-15 co-immunized group secreted IFN-γ, while 0.12% ± 0.13% antigen-specific CD8$^+$ T cells in pHBc144 and pcDNA3 co-immunized group were IFN-γ$^+$ CD8$^+$ T cells (Figure 5B, Figure 6 C and D). The antigen-specific memory cells still could be detected directly *ex vivo* in the circulation on d 90 post-boost (Figure 5B, Figure 6D).

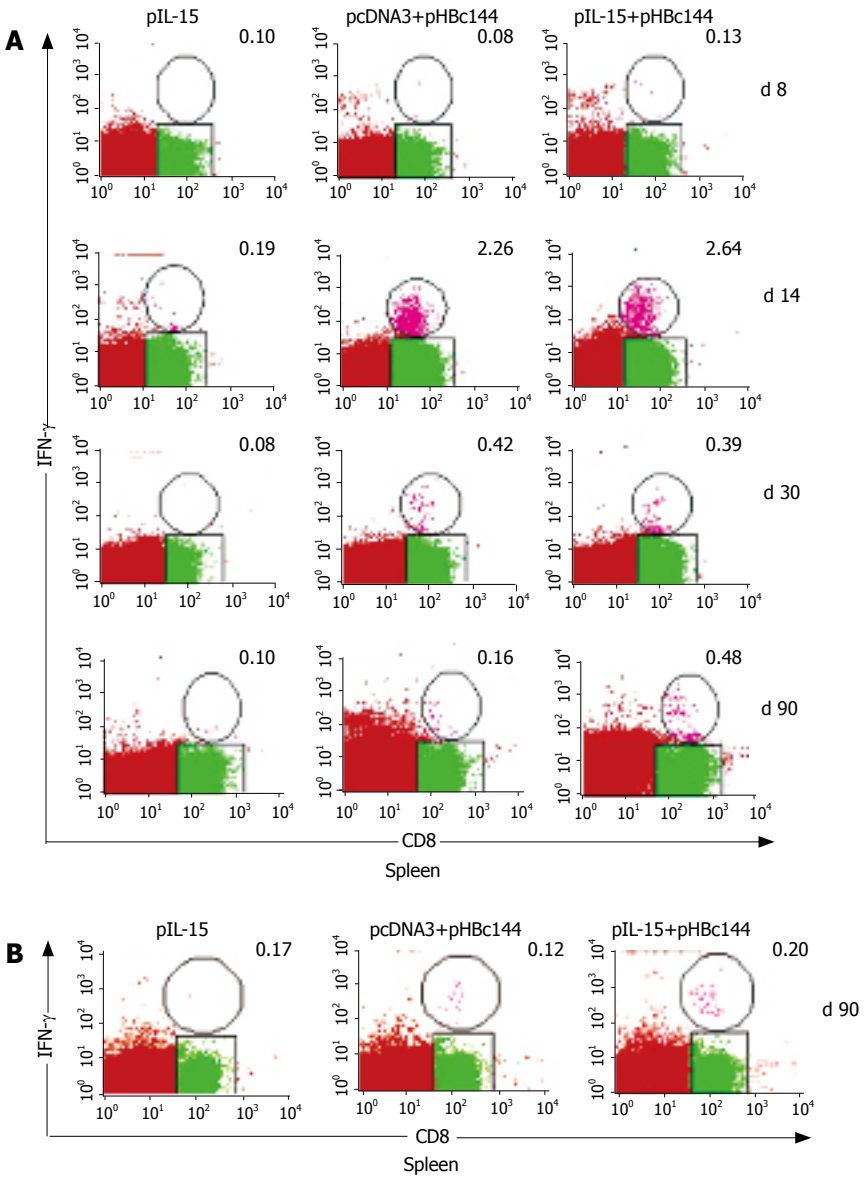
HBcAg specific CD4/CD8 in spleen and PBMCs induced by HBcDNA vaccine

At the same time point, we checked the total CD4$^+$ and CD8$^+$ cell percentage (Table 1), IL-15 had no big effect on the ratio of CD4$^+$ and CD8$^+$.

HBcAg specific humoral response induced by DNA vaccine

The HBcAg specific humoral immune response induced by pHBc144 was detected by ELISA for anti-HBc antibody in serum. The plates were read with Titerek Multiskan MCC/340 at A_{450}.The wells with an absorbance of ⩾ 0.1 (above background, 0.020) were scored as positive.

Four weeks after the first pHBc144 immunization, all mice underwent anti-HBc seroconversion. The mean titer of anti-HBc IgG in pHBc144 + pIL-15 group reached $2^{9.81} \pm 2^{0.9}$ on d 30. After being boosted, the mice produced a higher titer of anti-HBc IgG ($2^{9.81} \pm 2^{0.9}$) than that of the primary response, and anti-HBc maintained at that level ($2^{9.81} \pm 2^{0.9}$) on d 90. The titer of anti-HBc IgG2a in pHBc144 + pIL-15 group ($2^{9.81} \pm 2^{0.9}$) was slightly higher



than that of pHBc144 + pcDNA3 group ($2^{8.22} \pm 2^{0.9}$), but there was no significant difference ($P > 0.05$).

DISCUSSION

IL-15 plays a pivotal role in the development, survival, and activation of NK cells, NK-T cells and cytokine release various subsets of T and B cells[10-12]. IL-15 maintains the homeostasis of memory phenotype $CD8^+$ T cells[13]. The IL-15 response to infectious agents, such as viruses and other intracellular organisms, may represent a critical element in the host defense against these pathogens. Optimized IL-15 in combin with HIV-1gag DNA constructs results in a significant enhancement of Ag-specific $CD8^+$ T cell proliferation and IFN-gamma secretion, and strong induction of long-lived $CD8^+$ T cell responses[14]. pDNA encoding IL-15 is capable of elevating survival rates of HSV-1-infected mice when coinjected with 1 μg of gB pDNA[15]. IL-15 has been reported to have a profound effect on the augmentation of $CD8^+$ T cell response against murine *T. gondii* infection[16]. *Toxoplasma*-specific $CD8^+$ T cell immunity in mice is depleted over time, but can be rescued by IL-15 treatment. These findings suggest that IL-15 may be useful as an immune adjuvant given with vaccination to enhance its biologic efficacy.

IL-15 is predominantly regulated posttranscriptionally at the level of translation and translocation. Although Northern blot analysis can reveal widespread constitutive expression of IL-15 mRNA in a variety of tissues such as placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells, and monocytes, it is difficult to demonstrate IL-15 in supernatants of many cells that express such mRNA. This discordance between IL-15 mRNA expression and IL-15 protein production is regulated by multiple elements including 12 upstream AUGs of the 50 UTR, a 48-aa signal peptide, and the C-terminus of the mature protein. The IL-15 signal peptide is an important factor in the negative regulation of IL-15 protein expression. There are two alternative leader peptides, one with 48 aa and the other with 21 aa[17]. IL-15 associated with a short 21-aa signal peptide is not secreted but stored intracellularly, appearing in nuclear and cytoplasmic components[18]. The long 48-aa isoform of the signal peptide might function as a negative regulator of IL-15 generation. The total quantity of IL-15 generated (the sum of IL-15 retained within the cells) increases 17- to 20-fold when the IL-15

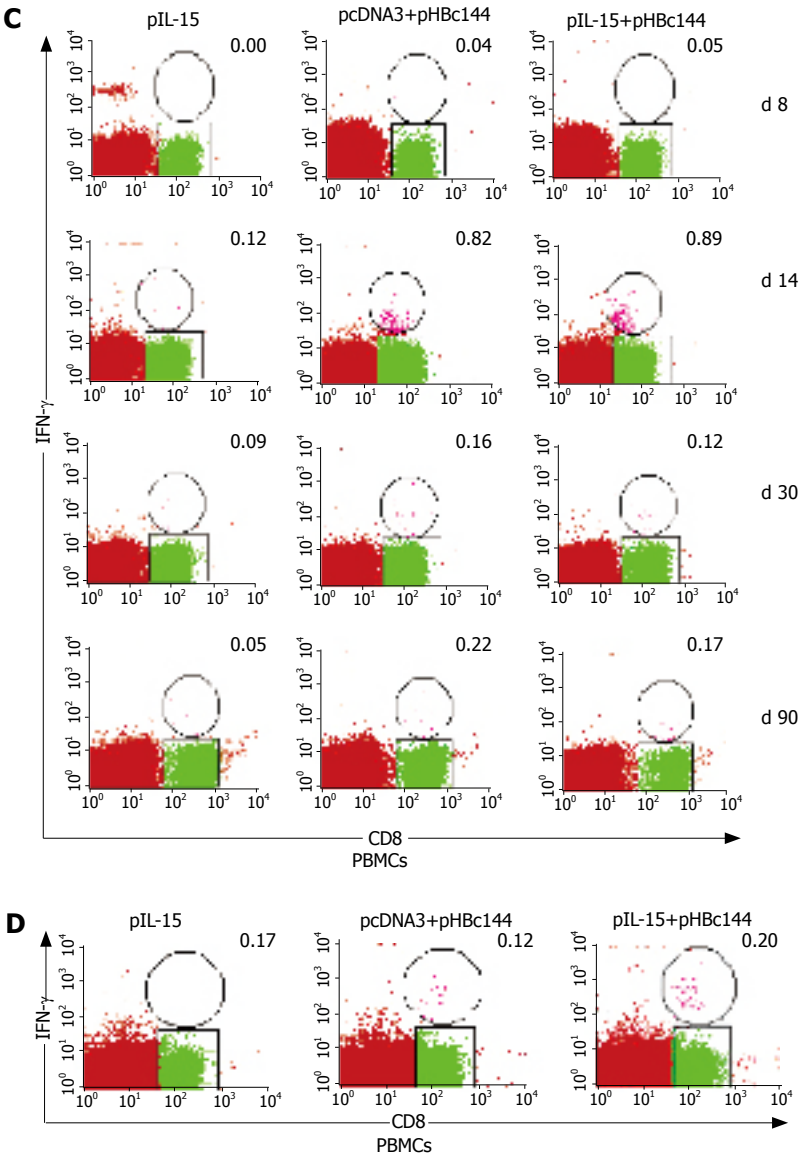


Figure 6 Percentages of HBc specific CD8$^+$ T cells in the spleen after primary (**A**) and secondary immunization (**B**) and in PBMCs after primary (**C**) and secondary immunization (**D**) detected by ICCs directly ex vivo at various time points. C57BL/6 mice were immunized with pHBc144 DNA or pIL-15 DNA and boosted at d 30 later. The splenocytes and harvested at various time points. Then the cells were cultured for 5 h in the absence or presence of the HBcAg peptide and double stained with anti-CD8a-PE and anti-IFN-γ-APC. CD8$^+$IFN-γ$^+$cells are enclosed in ovals, and the numbers in the upper right quadrants represent the percentage of CD8$^+$IFN-γ$^+$ cells to total CD8$^+$ T cells. The data from each group at each time point represent mean ± SD (n = 3). Naïve mice were used as negative controls. The cut-off value was 0.1%.

signal peptide is replaced by that of IL-2. Parallel studies showed that the quantity of IL-2 secreted is reduced 40- to 50-fold when COS cells are transfected with the reciprocal construct in which the IL-2 signal peptide is replaced with that of IL-15. Furthermore, after the IL-15 signal peptide is replaced with that of CD33 [19], translation and secretion increase, supporting the view that IL-15 expression is controlled posttranscriptionally at the level of translation and secretion.

We cloned the classical 48-aa signal peptide isoform of IL-15 (LSP-IL-15). When COS cells were transfected with the wild-type construct[19], the quantity of IL-15 protein was very low (360 pg per 200 000 cells). Moreover, in most cases it was difficult to demonstrate IL-15 in the culture supernatants. We could not detect IL-15 protein in culture supernatants by Western blot after transfecting COS cells with our construct (data not shown). We therefore subcloned the LSP-IL-15 cDNA into expression vector and let it translate in *E coli*. Western blot analysis showed a M_r 26 000 fusion protein, which was identified with NCBI sequence.

The IL-15 associated with the long 48-aa signal peptide enters the endoplasmic reticulum where it was glycosylated. Nevertheless, IL-15 is secreted after trafficking through the Golgi, yielding a cytokine with a fully processed signal peptide. We measured the biological function of the construct by detecting the IL-15 in the supernatant of transfection.

Meazza *et al*[20] and Onu *et al*[17] found that IL-15 is not secreted when IL-15 SP is employed. Nevertheless, Waldmann *et al*[21] assessed cultures from COS cells transfected with the wild-type IL-15 coding sequence at 120 h and found that at least 90% of the IL-15 protein (though at low levels) can be identified in the culture

supernatants, and 10% is retained within the cells, as evaluated by sensitive ELISA assays. Our result is consistent with that of Waldmann *et al*. Although in COS cells transfected with IL-15 expression construct, the levels of IL-15 protein synthesized and secreted were very low (3 logs less than those obtained with a comparable IL-2 construct), LSP-IL-15 could secrete in the supernatant of COS cells transfected with IL-15 expression construct and have bioactivity, and can induce CTLL-2 cell proliferation. This difference between the different studies may be due to the fact that the CTLL-2 strain cannot respond to IL-15.

To determine if the IL-15 plasmid has many effects on the humoral and cellular immune response, especially $CD8^+$ T cell response induced by DNA vaccination, splenocytes and PBMCs from the pIL-15 and HBcAg DNA vaccine co-immunized mice were evaluated for IFN-γ expression on antigen-specific $CD8^+$ T cells and anti-HBc IgG in serum was detected at each time point. We selected them for the DNA plasmid format because they are inexpensive, noninfectious and can be readily optimized to enhance immunogenicity and protective efficacy. Furthermore, DNA vaccines can induce robust and durable $CD8^+$ T cell responses, and can be used to deliver a large number of CTL epitopes[22]. Our results showed that pIL-15 had no effect on anti-HBc IgG induced by pHBc144. Although antigen-specific $CD8^+$ T cells reached a peak on d 14 after priming, pIL-15 had no effect on antigen-specific $CD8^+$ T cells in splenocytes or PBMCs induced by HBcAg DNA vaccine. pIL-15 could increase the percentage of antigen-specific $CD8^+$ T cells induced by HBcAg DNA vaccine on d 90 after priming and boosting. These two time points belonged to memory phase of immune response.

The existence of the basic effector Th1 and Th2 subsets is now well accepted, and used to plan therapeutic and vaccine strategies. Th1 cells produce interlukin 2 (IL-2), interferon γ and lymphotoxin (LT), whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-13[23]. The function of Th1 and Th2 cells correlates well with their distinctive cytokines. Th1 cells are involved in cell-mediated inflammatory reactions. Several Th1 cytokines activate cytotoxic and inflammatory functions, while Th1 clones induce delayed-type hypersensitivity (DTH) reactions; and IFN-γ is commonly expressed at sites of DTH reactions Th2 cytokines promote antibody production, particularly IgE responses, and also enhance eosinophil proliferation and function. Accordingly, Th2 cytokines are commonly found in association with strong antibody and allergic responses. Unfortunately, we did not evaluate the effect of IL-15 on antigen specific Th1 cells induced by HBc DNA vaccine. But our results showed that IL-15 had no effect on the ratio of $CD4^+$ and $CD8^+$.

Chronic HBV infection is one of the most common infectious diseases worldwide and leads to a high morbidity and mortality due to the development of liver cirrhosis and cancer. Virus persistence is believed to be due to an insufficient antiviral Th cell and CTL response of the host[24]. Effective HBV-specific $CD8^+$ T cell responses have been proven to inhibit virus replication independently of liver damage[25,26].

During HBV replication, the nucleocapsid or core particle recruits into the pregenomic RNA and viral reverse transcriptase, and provides the template on which the surface envelope components assemble. Recombinant M_r 21 000 (P21) core protein self-assembles into 28-nm particles representing the native hepatitis B core Ag (HBc Ag)[27]. Since it plays a central role in nucleocapsid formation and pregenomic viral RNA packaging, HBV core antigen represents another interesting target for DNA-based vaccine, although less data are available on DNA immunization to core antigen protein compared to the envelope protein. DNA-based immunization of mice to the HBV core has been shown to efficiently prime specific antibody and cytotoxic-T-lymphocyte responses[28].

Interferon is one of the most effective drugs for chronic HBV infection[29]. Experiments with HBV transgenic mice suggest that intrahepatic secretion of antiviral cytokines, such as IFN-γ and TNF-α by CTLs and Kupffer cells can interrupt the HBV life cycle without lysis of infected hepatocytes. The rapid reduction of HBV-DNA before hepatocellular damage in acute infections in chimpanzees and humans suggests that a similar non-cytolytic mechanism is, at least in part, responsible for inhibition of viral replication during natural infection[30].

In conclusion, DNA vaccine constructed by HBc Ag 1-144 amino acid induces effective cell immunity, cytokine plasmid-delivered IL-15 enhances $CD8^+$ T cell longevity, IL-15 is a possible candidate adjuvant for HBV DNA vaccine.

REFERENCES

1 **Yajima T**, Nishimura H, Sad S, Shen H, Kuwano H, Yoshikai Y. A novel role of IL-15 in early activation of memory CD8+ CTL after reinfection. *J Immunol* 2005; **174**: 3590-3597

2 **Schluns KS**, Lefrançois L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 2003; **3**: 269-279

3 **Umemura M**, Nishimura H, Saito K, Yajima T, Matsuzaki G, Mizuno S, Sugawara I, Yoshikai Y. Interleukin-15 as an immune adjuvant to increase the efficacy of Mycobacterium bovis bacillus Calmette-Guérin vaccination. *Infect Immun* 2003; **71**: 6045-6048

4 **Toka FN**, Rouse BT. Mucosal application of plasmid-encoded IL-15 sustains a highly protective anti-Herpes simplex virus immunity. *J Leukoc Biol* 2005; **78**: 178-186

5 **Lazarevic V**, Yankura DJ, DiVito SJ, Flynn JL. Induction of Mycobacterium tuberculosis-specific primary and secondary T-cell responses in interleukin-15-deficient mice. *Infect Immun* 2005; **73**: 2910-2922

6 **Yang L**, Liu J, Kong YY, Wang Y, Li GD. DNA vaccine of the fusion gene of the core region of HBV and HCV. *Zhongguo Kexue* 1999; **29**: 246-252

7 **Sambrook J**, Russell DW. Molecular Cloning: a laboratory manual. Third Edition. New York: Cold Spring harbor laboratory Press, 2001: 14-20

8 **Mire-Sluis AR**, Thorpe R. Laboratory protocols for the quantitation of cytokines by bioassay using cytokine responsive cell lines. *J Immunol Methods* 1998; **211**: 199-210

9 **Lu M**, Isogawa M, Xu Y, Hilken G. Immunization with the gene expressing woodchuck hepatitis virus nucleocapsid protein fused to cytotoxic-T-lymphocyte-associated antigen 4 leads to enhanced specific immune responses in mice and woodchucks. *J Virol* 2005; **79**: 6368-6376

10 **Yajima T**, Nishimura H, Ishimitsu R, Watase T, Busch DH, Pamer EG, Kuwano H, Yoshikai Y. Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following

a microbe exposure. *J Immunol* 2002; **168**: 1198-1203
11 **Alpdogan O**, van den Brink MR. IL-7 and IL-15: therapeutic cytokines for immunodeficiency. *Trends Immunol* 2005; **26**: 56-64
12 **Diab A**, Cohen AD, Alpdogan O, Perales MA. IL-15: targeting $CD8^+$ T cells for immunotherapy. *Cytotherapy* 2005; **7**: 23-35
13 **Klonowski KD**, Lefrançois L. The CD8 memory T cell subsystem: integration of homeostatic signaling during migration. *Semin Immunol* 2005; **17**: 219-229
14 **Kutzler MA**, Robinson TM, Chattergoon MA, Choo DK, Choo AY, Choe PY, Ramanathan MP, Parkinson R, Kudchodkar S, Tamura Y, Sidhu M, Roopchand V, Kim JJ, Pavlakis GN, Felber BK, Waldmann TA, Boyer JD, Weiner DB. Coimmunization with an optimized IL-15 plasmid results in enhanced function and longevity of CD8 T cells that are partially independent of CD4 T cell help. *J Immunol* 2005; **175**: 112-123
15 **Cui FD**, Asada H, Jin ML, Kishida T, Shin-Ya M, Nakaya T, Kita M, Ishii M, Iwai M, Okanoue T, Imanishi J, Mazda O. Cytokine genetic adjuvant facilitates prophylactic intravascular DNA vaccine against acute and latent herpes simplex virus infection in mice. *Gene Ther* 2005; **12**: 160-168
16 **Khan IA**, Moretto M, Wei XQ, Williams M, Schwartzman JD, Liew FY. Treatment with soluble interleukin-15Ralpha exacerbates intracellular parasitic infection by blocking the development of memory CD8+ T cell response. *J Exp Med* 2002; **195**: 1463-1470
17 **Onu A**, Pohl T, Krause H, Bulfone-Paus S. Regulation of IL-15 secretion via the leader peptide of two IL-15 isoforms. *J Immunol* 1997; **158**: 255-262
18 **Gaggero A**, Azzarone B, Andrei C, Mishal Z, Meazza R, Zappia E, Rubartelli A, Ferrini S. Differential intracellular trafficking, secretion and endosomal localization of two IL-15 isoforms. *Eur J Immunol* 1999; **29**: 1265-1274
19 **Bamford RN**, DeFilippis AP, Azimi N, Kurys G, Waldmann TA. The 5′ untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control. *J Immunol* 1998; **160**: 4418-4426
20 **Meazza R**, Gaggero A, Neglia F, Basso S, Sforzini S, Pereno R, Azzarone B, Ferrini S. Expression of two interleukin-15 mRNA isoforms in human tumors does not correlate with secretion: role of different signal peptides. *Eur J Immunol* 1997; **27**: 1049-1054
21 **Tagaya Y**, Kurys G, Thies TA, Losi JM, Azimi N, Hanover JA, Bamford RN, Waldmann TA. Generation of secretable and nonsecretable interleukin 15 isoforms through alternate usage of signal peptides. *Proc Natl Acad Sci USA* 1997; **94**: 14444-14449
22 **Wilson CC**, McKinney D, Anders M, MaWhinney S, Forster J, Crimi C, Southwood S, Sette A, Chesnut R, Newman MJ, Livingston BD. Development of a DNA vaccine designed to induce cytotoxic T lymphocyte responses to multiple conserved epitopes in HIV-1. *J Immunol* 2003; **171**: 5611-5623
23 **Rajcáni J,** Mosko T, Rezuchová I. Current developments in viral DNA vaccines: shall they solve the unsolved? *Rev Med Virol* 2005; **15**: 303-325
24 **Zheng BJ**, Zhou J, Qu D, Siu KL, Lam TW, Lo HY, Lee SS, Wen YM. Selective functional deficit in dendritic cell--T cell interaction is a crucial mechanism in chronic hepatitis B virus infection. *J Viral Hepat* 2004; **11**: 217-224
25 **Wu Y**, Zhang J, Chen S, Chen A, Wang L, Li J, Zhao T, Zou L, Tang Y, Tingrong L, Wang F. Frequencies of epitope-specific cytotoxic T lymphocytes in active chronic viral hepatitis B infection by using MHC class I peptide tetramers. *Immunol Lett* 2004; **92**: 253-258
26 **Bertoletti A**, Maini M, Williams R. Role of hepatitis B virus specific cytotoxic T cells in liver damage and viral control. *Antiviral Res* 2003; **60**: 61-66
27 **Hacker HJ**, Deres K, Mildenberger M, Schröder CH. Antivirals interacting with hepatitis B virus core protein and core mutations may misdirect capsid assembly in a similar fashion. *Biochem Pharmacol* 2003; **66**: 2273-2279
28 **Xu W**, Chu Y, Zhang R, Xu H, Wang Y, Xiong S. Endoplasmic reticulum targeting sequence enhances HBV-specific cytotoxic T lymphocytes induced by a CTL epitope-based DNA vaccine. *Virology* 2005; **334**: 255-263
29 **Kanwal F**, Gralnek IM, Martin P, Dulai GS, Farid M, Spiegel BM. Treatment alternatives for chronic hepatitis B virus infection: a cost-effectiveness analysis. *Ann Intern Med* 2005; **142**: 821-831
30 **Tang TJ**, Kwekkeboom J, Laman JD, Niesters HG, Zondervan PE, de Man RA, Schalm SW, Janssen HL. The role of intrahepatic immune effector cells in inflammatory liver injury and viral control during chronic hepatitis B infection. *J Viral Hepat* 2003; **10**: 159-167

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RAPID COMMUNICATION

Hand-assisted laparoscopic surgery of abdominal large visceral organs

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Abstract

AIM: To design a hand-assisted laparoscopic approach in an attempt to provide an option for laparoscopic resection of abdominal large viscera.

METHODS: A 5-6 cm incision (for HandPort) and 2 trocars were employed. The main vessel of the target organ was taken as a "core", and all tissues around the core were taken as peripheral structures. The peripheral structures were dissected first, and the core vessels were treated last. Twenty-six patients underwent laparoscopic deroofing of the hepatic huge cysts, resection of the segments lying at the outer edge of the liver (segments 2 to 6), splenectomy, hemicolectomy, ileocecectomy and subtotal gastrectomy with HandPort device, harmonic scalpel, or Ligasure.

RESULTS: The duration of the procedure was within 2 hours. Blood loss amounted to 8-120 mL. The conversion rate was 3.8% (1/26). All patients had uneventful postoperative courses with less pain, earlier oral intake, and faster recovery, compared with conventional surgery.

CONCLUSION: This method combines the advantages of both open and laparoscopic techniques, achieving better hemostasis effect, shortening the operative time, and is beneficial to the patients.

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Key words: Minimally invasive surgery; Hand-assisted laparoscopic surgery; Hepatic segmentectomy; Hemicolectomy; Subtotal gastrectomy

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INTRODUCTION

Since Mouret performed the successful laparoscopic cholecystectomy in 1987, new frontiers have opened for minimally invasive surgery[1], and has been expanded to more extensive procedures such as resection of the abdominal large viscera[2-4]. The minimally invasive surgery is superior to conventional open surgery because it offers significant benefits to the patients including much shorter incision, less pain and faster recovery. Despite instrumentation has dramatically improved over the past decade, laparoscopic resection of abdominal large organ still has several concerns regarding the safety, technical difficulties, rapidity and complications such as bleeding, injury to major adjacent structures, which affect the short-term and long-term outcomes[5].

In an effort to largely simplify and shorten the procedure, provide an easier, safer approach, and decrease patients' postoperative pain and stay, we have designed a method for laparoscopic resection of abdominal large viscera. Usually, several 5-12 mm trocars are placed in the abdominal wall in laparoscopic surgery. However, there must be 5-6 cm port for extraction of a resected specimen in removal of abdominal large visceral organ. From "1 port + 2 trocars" as the minimal invasive wounds, we used a quick and safe hand-assisted laparoscopic technique for resection of abdominal large viscera, by taking advantages of both open and laparoscopic techniques.

MATERIALS AND METHODS

Patients

From May 2001 to December 2002, 26 patients, 15 males and 11 females with a mean age of 61.8 yeas (range 42-71 years), underwent hand-assisted laparoscopic surgery involving resection of liver, spleen, colon, jejunum, ileocecum, stomach and gallbladder, including 2 emergency operations: a 71 year-old female had uncontrolled bleeding from multiple diverticulosis of ascending colon, and another 54 year-old female suffered from carcinoma of terminal ileum associated with ileus. The procedure was carried out endocorporeally and extracorporeally, by means of the HandPort system (Smith & Nephew Inc. Andover, Massachusetts), harmonic scalpel (HCS 15 and HCS C5, Ethicon, Endo-surgery, Cincinnati, Ohio. Ultra shears 5 mm instrument, US Surgical, Tyco Healthcare) or Ligasure(10 mm Atlas, Vallylab, Boulder, Tyco Healthcare). All procedures were performed with the patients in the

Table 1 (HALS) operations and indicating diseases (26 cases)

Cases	Operations	Indicating diseases
1-2	Left lateral segmentectomy	Hepatitis B-related cirrhosis supervened hepatocellular carcinoma (HCC), hepatic cavernous hemangioma
3	S 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC
4	S 5 and 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC
5	S 5, 6 and partial 4 segmentectomy + cholecystectomy	Hepatitis B-related cirrhosis supervened HCC
6	Partial S 4 segmentectomy	Hepatic cavernous hemangioma
7-11	Deroofing of the hepatic huge cysts	The 4, 6, 7, 8 segmental hepatic cysts; the 4, 5, 6, 7, 8 segmental hepatic cysts; the 5, 6, 7 segmental hepatic cysts (2); the 5, 6, 7 segmental hepatic cysts with calcification of cystic wall
12-15	Right hemicolectomy	Carcinoma of the ascending colon (3) [1]Multiple diverticulosis of ascending colon with massive hemorrhage
16	Left hemicolectomy + partial jejunectomy	Carcinoma of the splenic flexure of colon with metastasizing to liver and jejunum,
17	Left hemicolectomy	[2]Carcinoma of the sigmoid with metastasizing to left ureter and common iliac artery
18-19	Ileocecectomy	[1]Carcinoma of the terminal ileum with ileus, multiple diverticulosis of the ileocecum and chronic appendicitis
20-21	Splenectomy	Idiopathic thrombocytopenic purpura, carcinoma of pancreatic body and tail
22-25	Subtotal gastrectomy	Duodenal ulcer (2), gastric ulcer, gastric ulcer with massive hemorrhage
26	Half gastrectomy + cholecystectomy	Diverticulitis of duodenum and gall stone

[1] Emergency operation; [2]Conversion to open operation.

Table 2 Location of the ports and positioning of the operator

Operations	HandPort	Camera[1]	Scalpel[1]	Operator[2]
Left hepatic lateral segmentectomy	R upper transrectus[3]	Umbilicus	L upper quadrant	R
Right hepatic segmentectomy	R pararectus abdominis	Umbilicus	L upper quadrant	R
Deroofing of the hepatic huge cyst	R subcosta	Umbilicus	L upper quadrant	L
	R upper transrectus	Umbilicus	L upper quadrant	L
	R pararectus abdominis	Umbilicus	L upper quadrant	R
Splenectomy	Upper midline[4]	Umbilicus	L umbilicus level	R
	L upper transrectus	Umbilicus	L lower umbilicus level	R
Right hemicolectomy	Upper midline	Umbilicus	Lower midline	R
Left hemicolectomy + partial jejunectomy	Upper midline	Umbilicus	Lower midline	R
Subtotal gastrectomy	Upper midline	Umbilicus	L lower umbilicus level	R
Half gastrectomy +cholecystectomy	Upper midline	Umbilicus	R lower umbilicus level	R
Ileocecectomy	Lower midline[3]	Umbilicus	R middle quadrant	L

[1]Camera and Scalpel can change places with each other during operation; [2]Left hand into abdomen, operator can change places during operation; [3]Two incisions for HandPort device were at the previous wounds of right upper transrectus abdominis and lower midline involved open partial gastrectomy and caesarean operation.respectively; [4]Just under the xiphoid process; R: right, L: left.

supine position, rotated appropriately, or with rolls under the flank of the appropriate side. The operations and indicating diseases are listed in Table 1.

The location of HandPort device was preferred to be directly over or close to the main (core) vessels of the target organ, in order to provide direct visualization to transect the core vessels or dissect the radical lymph notes. The location of ports and positioning of the operator are listed in Table 2.

Before placement of HandPort device

A 5-6 cm abdominal incision was performed, before placement of HandPort device to allow for introduction of the hand to assist in placing two trocars, similar to placement of abdominal drains in open surgery, and also to allow the hands to perform abdominal exploration. If the dissection preceded the resection, the following procedures were done under direct vision, including mobilizing intraperitoneal adhesion, dissecting the peripheral structures, or placing a loop for occlusion of hepatic hilar pedicles, in case of emergency use.

Following placement of HandPort device

The HandPort device was placed, with the abdomen insufflated under a pressure of 13-14 mmHg. The left hand was reintroduced. While exposing and retracting or pulling tissues with the hand, the peripheral structures including adhesions, peritoneum, ligaments, omentum, mesentery, as well as the hepatic parenchyma around the "core" pedicles were dissected by harmonic scalpel or Ligasure, occasionally by combined use of electrocoagulation without any laparoscopic suture or ligation.

Treatment of the core vessels finally

Once the target organ was mobilized, its core vessels were treated openly through the port incision by conventional open technique or vascular endo-stapler, as the location of the port incision was most direct over or close to the core vessels of the target organ.

Among our patients, a 71 year-old woman suffered

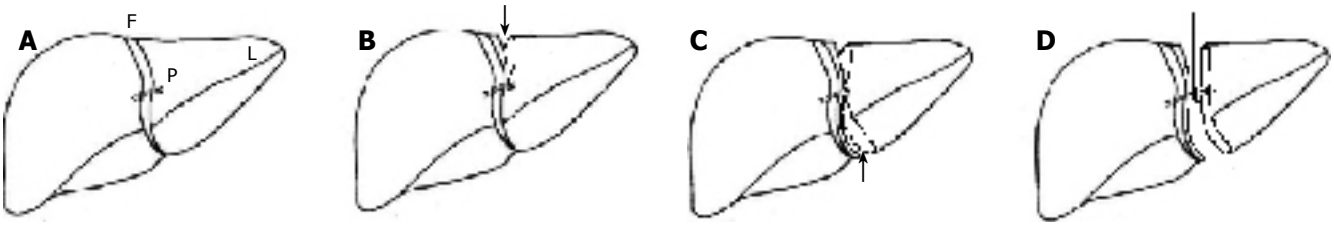

Figure 1 **A**: F, falciform ligament; P, portal pedicles; L, lateral segment; **B**: Dissecting from top to bottom till down to core vessels; **C**: Dissecting from bottom to top till up to core vessels; **D**: Transecting core vessels openly using an endo-stapler.

Table 3 Indications for conventional surgery (25 cases)

Cases	Operations	Indications
1-2	Left lateral segmentectomy	Hepatitis B-related cirrhosis supervened HCC, hepatic cavernous hemangioma
3-6	Deroofing of the hepatic huge cysts	Right hepatic huge cysts (4)
7-8	Hepatic S 5 and 6 segmentectomy	Hepatic cavernous hemangioma, hepatitis B-related cirrhosis supervened HCC
9-10	Hepatic S 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC, hepatic cavernous hemangioma
11	Hepatic S 5 segmentectomy + cholecystectomy	Hepatitis B-related cirrhosis supervened HCC
12-13	Splenectomy	Splenic infarction, splenic cysts
14-17	Right hemicolectomy	Carcinoma of the ascending colon (3), multiple polyps of ascending colon
18	Left hemicolectomy	Carcinoma of the descending colon
19-20	Ileocecectomy	Carcinoma of the terminal ileum, multiple diverticulosis
21-25	Subtotal gastrectomy	Duodenal ulcer(2), [1]gastric ulcer with bleeding, gastric ulcer, diverticulitis of duodenum with bleeding

[1]Emergency operation.

Table 4 HALS compared with conventional open surgery

	Cases	Incision (cm)	Time of operation (min)	Operative bleeding (mL)	Postoperative feeding (d)	Hospital stay (d)
Open surgery	25	16-33	125-240	80-900	2-6	7-26
(mean)		22.5	190	340	4	13
HALS	25[1]	5-6	30-120	8-120	1-3	4-15
(mean)		5.3	86	85	1.8	7

[1]One of the 26 patients converting to open operation was not included. Statistical comparison was performed using the Independent-Samples *t*-Test ($P < 0.001$).

from hepatitis B-related cirrhosis supervened HCC and arrhythmia. She had had an open partial gastrectomy 5 years ago due to leiomyoma of stomach. She underwent hand-assisted laparoscopic left lateral segmentectomy. The HandPort incision of 6 cm in length was located at the previous wound scar at the right upper transrectus abdominis (the previous incision of 18 cm in length). The strategy of the procedure was to treat the portal pedicles of the left lateral segment as core vessels. First, the adhesions between wound peritoneum and omentum, liver and stomach were dissected. A loop was then placed around hepatic hilar pedicles, in case of requiring interrupting hepatic afferent blood flow. Two trocars for camera and harmonic scalpel were introduced by the open method of making abdominal drains. Following placement of HandPort system, the left hand was reintroduced and the target segment retracted. Ligament attachments of the left lobe were divided until the left lateral segment was completely freed, as shown in Figure 1A. The peripheral parenchyma around the core vessels was dissected from top toward bottom down to the core vessels (portal pedicles) with a harmonic scalpel (HCS 15 from Ethicon, Cincinati) (Figure 1B). Then, dissection switched from the bottom toward top up to the core vessels. The core vessels were completely freed (Figure 1C). Finally, the core vessels were transected by a vascular endo-stapler through the port incision openly (Figure 1D). The resected specimen was extracted immediately. There was no active blood oozing from the resected raw surface of the liver, which was covered with absorbable hemostasis gauze. The blood loss was less than 60 mL, without temporary inflow occlusion during operation. There was no need for abdominal drain. The resected left lateral segment was 15 cm × 8 cm × 4 cm.

RESULTS

Twenty-six patients underwent uneventful operation courses. One patient was converted to open procedure because his left ureter and common iliac artery were invaded by the sigmoid cancer. The conversion rate was 3.8% (1/26). The length of the procedures was under 2 hours (range 30 min-2 h). The operative blood loss was between 8 to 120 mL. There were no postoperative complications except that a 70 year-old man with chronic pulmonary emphysema and carcinoma of ascending colon, who underwent hand-assisted laparoscopic right hemicolectomy, developed 2 split stitches at the wound because of a violent cough on the 2nd postoperative day, and another patient had a wound stitch reaction. After operation, all patients felt less pain, resumed earlier oral intake of food, and faster recovery, compared with 25

consecutive corresponding cases of conventional surgery. The data are summarized in Tables 3 and 4.

DISCUSSION

Compared with full minimal laparoscopic large organ resection, an incision is often required for extraction of the resected specimen. In fact, there are often "1 port + more than 2 trocars" abdominal wounds in full minimal laparoscopic large viscera resection. By use of currently available tools including HandPort system, harmonic scalpel, Ligasure[6], we designed "1 port + 2 trocars" specific HALS for safe and quick resection of large abdominal visceral organs.

This technique can be of real significance in avoidance of vascular bleeding during removal of the target organ, because the final step is transection of the core vessels. There is no need for worrying about bleeding as the staplers are taken off. Furthermore, a hand inside the abdomen can play an important role in preventing or controlling bleeding. On the contrary, for total laparoscopic operation, which were several times higher in blood loss and longer in operative time, the unmanageable bleeding has become the main cause to convert to open surgery. Total laparoscopic operation does not equal to the minimally invasive surgery. The minimally invasive surgery presents not only the minimal abdominal wound, but also the minimal surgical intervention and complications including bleeding, injury to major adjacent structures, conversion to open surgery and operative time. The technique we employed is by all means the small ports to accomplish the big organ resection with minimal surgical stress, better hemostasis and shorter operative time, and faster recovery of the patients.

An excellent combination of laparoscopic and open techniques for hemicolectomy

Hand-assisted laparoscopic hemicolectomy by this technique is presumed to be an excellent approach combining advantages of laparoscopic and open surgeries. The procedure includes: (1) Laparoscopically, the distal peripheral structures are mobilized by harmonic scalpel or Ligasure with hand-assistance; (2) Conventionally, the mobilized target colon is long enough to be extracted through the optimal base retractor of HandPort system, through which the major core vessel pedicles are transected, or the radical lymph nodes are dissected under direct vision, then the specimen is removed and delivered to prevent port side "seeding"; (3) Extracorporeally, end to end coloileostomy or colocolostomy is performed by sutureless biofragmentable anastomosis ring (BAR), or single layer anastomosis.

In our series, the HALS wound length of 5-6 cm (mean 5.3 cm) was much shorter than the open surgical wound length of 16-33 cm (mean 22.5 cm). Other studies have reported full laparoscopic wound length of 8.5 cm[7]. The operative time in our group was under 2 h. In addition, previous studies revealed mean operative times for full laparoscopic colectomy, converted procedures, right, and sigmoid resection were 164, 203, 121 and 177 min, respectively[8].

Advantages over full laparoscopic splenectomy

Our specifically designed HALS in splenectomy has advantage over total laparoscopic splenectomy. First, through the small incision port, the gastrosplenic ligament was dissected as much as possible by open technique before pneumoperitoneum. Second, after placement of the HandPort device, a hand into the abdomen assisted harmonic scalpel or Ligasure to dissect the rest ligaments. It seemed the spleen was retracted by hand with more safety. It also provided better exposure, easier and faster performance than other instruments. Third, splenic pedicle was transected by endo-stapler laparoscopically, or through the small incision with open visualization. Lastly, the resected specimen was extracted immediately after removal of the spleen. However, in full laparoscopic splenectomy with about 4 trocars, the specimen is bagged, one port incision is extended, and the spleen is broken into pieces, and then delivered.

A definite advantage in terms of HALS in hepatectomy

Our study demonstrates HALS in resection of the segments lying at outer edge of the liver (segments 2 to 6) can be performed with ease, safety and rapidity. Placement of the hand into the abdomen plays an important role, because there is no ideal instrument available for grasping the liver but the hand, which acts as a comparatively atraumatic grasping clamp and retractor providing good exposure, and also allows for fingers to identify major pedicles after finishing parenchymal division with Ligasure or harmonic scalpel. The major pedicles are transected using vascular endo-stapler under direct vision through the small incision, and the specimen is delivered at once without breaking.

Advantages over full laparoscopic subtotal gastrectomy

First, the greater and lesser omentum are dissected using this technique much easier and quicker. Second, the stomach is transected and gastrojejunostomy is performed by using open staplers, which are much easier, quicker, more reliable, and less expensive than using endo-staplers in full laparoscopic subtotal gastrectomy. Third, a big and hard duodenal ulcer like the one in our cases is not the indication for full laparoscopic technique, because such duodenal ulcer can neither be transected, nor can the stump be closed with staplers.

The design of the approach for HALS in resection of abdominal large viscera is based on the minimal abdominal wound "1 port + 2 trocars", as well as the minimal surgical intervention, combining the laparoscopic benefits with the advantages of a conventional open technique. Even though the target organs include parenchymatous and hollow viscera, the rules of resection are the same, which allow for first dissecting the peripheral structures, and finally transecting the core vessel pedicles. This technique is minimal in access, safe, and rapid in laparoscopic resection of abdominal large viscera.

REFERENCES

1 **Perissat J**, Vitale GC. Laparoscopic cholecystectomy: gateway to the future. *Am J Surg* 1991; **161**: 408

2 **Tuech JJ**, Pessaux P, Regenet N, Rouge C, Hennekinne S, Bergamaschi R, Arnaud JP. Laparoscopic colectomy for sigmoid diverticulitis: a prospective study in the elderly. *Hepatogastroenterology* 2001; **48**: 1045-1047
3 **Kusminsky RE**, Boland JP, Tiley EH, Deluca JA. Hand-assisted laparoscopic splenectomy. *Surg Laparosc Endosc* 1995; **5**: 463-467
4 **Kusminsky RE**, Boland JP, Tiley EH. Hand-assisted laparoscopic surgery. *Dis Colon Rectum* 1996; **39**: 111
5 **Litwin DE**, Darzi A, Jakimowicz J, Kelly JJ, Arvidsson D, Hansen P, Callery MP, Denis R, Fowler DL, Medich DS, O' Reilly MJ, Atlas H, Himpens JM, Swanstrom LL, Arous EJ, Pattyn P, Yood SM, Ricciardi R, Sandor A, Meyers WC. Hand-assisted laparoscopic surgery (HALS) with the HandPort system: initial experience with 68 patients. *Ann Surg* 2000; **231**: 715-723
6 **Msika S**, Deroide G, Kianmanesh R, Iannelli A, Hay JM, Fingerhut A, Flamant Y. Harmonic scalpel in laparoscopic colorectal surgery. *Dis Colon Rectum* 2001; **44**: 432-436
7 **Brown SR**, Eu KW, Seow-Choen F. Consecutive series of laparoscopic-assisted vs. minilaparotomy restorative proctocolectomies. *Dis Colon Rectum* 2001; **44**: 397-400
8 **Lauter DM**, Froines EJ. Initial experience with 150 cases of laparoscopic assisted colectomy. *Am J Surg* 2001; **181**: 398-403

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RAPID COMMUNICATION

Treatment of hepatoma with liposome-encapsulated adriamycin administered into hepatic artery of rats

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Abstract

AIM: To observe the therapeutic effects of liposome-encapsulated adriamycin (LADM) on hepatoma in comparison with adriamycin solution (FADM) and adriamycin plus blank liposome (ADM + BL) administered into the hepatic artery of rats.

METHODS: LADM was prepared by pH gradient-driven method. Normal saline, FADM (2 mg/kg), ADM+BL (2 mg/kg), and LADM (2 mg/kg) were injected *via* the hepatic artery in rats bearing liver W256 carcinosarcoma, which were divided into four groups randomly. The therapeutic effects were evaluated in terms of survival time, tumor enlargement ratio, and tumor necrosis degree. The difference was determined with ANOVA and Dunnett test and log rank test.

RESULTS: Compared to FADM or ADM + BL, LADM produced a more significant tumor inhibition (tumor volume ratio: 1.243 ± 0.523 *vs* 1.883 ± 0.708, 1.847 ± 0.661, $P < 0.01$), and more extensive tumor necrosis. The increased life span was prolonged significantly in rats receiving LADM compared with FADM or ADM+BL (231.48 *vs* 74.66, 94.70) ($P < 0.05$).

CONCLUSION: The anticancer efficacies of adriamycin on hepatoma can be strongly improved by liposomal encapsulation through hepatic arterial administration.

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Key words: Adriamycin; Liposome; Hepatoma

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INTRODUCTION

Adriamycin (ADM) is extensively used to treat patients with hepatocellular carcinoma (HCC). *In vitro* studies have verified that the cytocidal effect of ADM depends on the concentration and duration of exposure[1]. Unfortunately, intravenous administration (iv) of a high-dose ADM is often associated with acute toxicity, such as myelosuppression, immunosuppression, and dose-cumulative cardiotoxicity. As a result, drug concentrations in hepatomas are limited. During the past few years, many researchers have reported on drug delivery systems for cancer chemotherapy that aim at the specific targeting of antitumor agents to tumor cells or tumor tissues, thus enhancing the efficacy of chemotherapy as well as reducing its toxicity[2]. Among them, liposomes have drawn much attention for their excellent bioavailability, biodegradability, and targeting, characteristically, to the reticuloendothelial system (RES), especially the liver and spleen[3].

On the other hand, liver primary tumors receive approximately 90% of their blood supply from the hepatic artery. Therefore, transhepatic arterial chemotherapy (TAC) is now widely recognized as an effective means for the treatment of HCC. With TAC, a much higher drug concentration in the tumor-residing zone can be achieved. It was reported that administration of ADM via the hepatic artery (ia) was able to increase the tumor ADM concentration three-fold compared to intravenous (iv) administration[4]. In patients with cancers, the ia administration of ADM reduced the plasma AUC by about 30%[5]. On the basis of these evidences, we hypothesized that a further significant therapeutic effect could be expected by combining TAC and liposome techniques. The current study was carried out to observe the therapeutic effects of liposome-encapsulated adriamycin (LADM) on hepatoma in comparison with the treatment results of adriamycin solution (FADM) and adriamycin plus blank liposome (ADM + BL) administered into the hepatic artery in rats.

MATERIALS AND METHODS

Liposomal adriamycin (LADM) preparation and characterization

Large unilamellar vesicles (LUVs) were prepared by the extrusion method described by Hope *et al*[6]. Appropriate amounts of lipid mixtures (EPC/Chol, 55:45 mol/mol) in chloroform were dried under a stream of nitrogen gas to form a homogeneous lipid film. The trace amount of solvent was then removed under vacuum overnight. The lipid film was hydrated in a low pH citrate buffer (pH 4.0, 300 mmol/L) by vortex mixing. The resulting multilamellar vesicles (MLVs) were frozen/thawed (liquid nitrogen/55℃) 5 times and extruded 10 times at 55℃ through two stacked 100 nm polycarbonate filters (Nuclepore) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, BC, Canada). ADM was then encapsulated by pH gradient-driven method as described previously[7].

The final product looked like a reddish, semitransparent, colloidal solution. Under electron microscope, LADM showed a global, regular contour with homogeneous size and distribution. The average 120 nm diameters of the liposomes were determined by quasi-elastic light scattering (QELS) using a Nicmp 370 submicrom particle sizer (Sante Burbara, CA, USA) as shown in Figure 1. EPC and Chol were quantified by high performance liquid chromatography (HPLC) method (Table 1). HPLC analysis was performed using a Perkin Elemer HPLC with a PE 200 LC pump, a PE ISS 200 Advanced LC sample Processor, PE 900 Series Interface and an Evaporative Light Scattering Detector (ELSD, Shimadzu). A YMC, PVA 5 m normal phase column was used in this study. The chromatograph was run in a gradient mode at a flow rate of 1 mL/min with the following program, where solution A contained chloroform-isopropanol (50:50, v/v) and solution B contained chloroform-isopropanol-water (36:55:9, v/v).

The drug-to-lipid ratio was 0.25. The drug-embedding ratio was more than 98%. Encapsulation efficiency was calculated as the percentage of ADM incorporated into liposomes relative to the initial amount of ADM in solution as shown in the equation below. The free ADM was separated by elution over a Sephadex G-50 column. Then the free ADM and liposomal ADM were determined by HPLC.

$$\text{Encapsulation efficiency (\%)} = \frac{(\text{adriamycin/lipid})_{\text{Encapsulated}}}{(\text{adriamycin/lipid})_{\text{total}}} \times 100$$

The liposomal particle sizes were identical before and after drug loading, and ADM encapsulated liposomes were very stable at room temperature for three days. There was no significant leakage of ADM from liposomes. Just before use, normal saline was added to adjust the final concentration of total ADM to 1.0 mg/mL. Free ADM (FADM) was prepared by dissolution of ADM in normal saline at the same concentration.

Animals and anesthesia

Sixty male Wistar rats weighing 230-270 g (mean, 250 g) were provided by Laboratory Animal Center of Fourth Military Medical University and randomly divided into 4 groups, 15 in each. Sumianxin (Changchun Agricultural Pastoral University) was used as anesthetic.

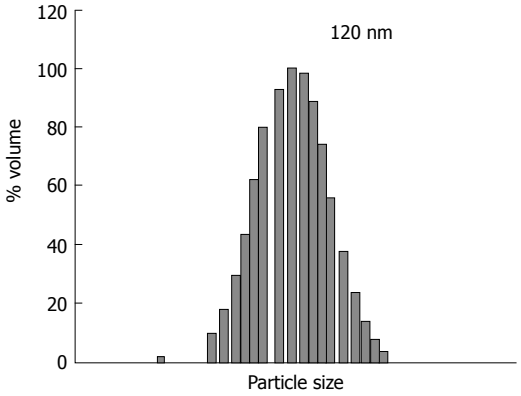

Figure 1 The particle size of prepared liposome with EPC/Chol at 55:45 molar ratio by the extrusion method. The average diameters were determined by QELS.

Table 1 Detailed solvent gradient program for analysis of EPC and Chol by HPLC

Time (min)	0-3	3-11	11-11.5	11.5-30
A%	100	0	0	100
B%	0	100	100	0

Solution A: Chloroform-isopropanol (50:50, v/v); Solution B: Chloroform-isopropanol-water (36:55:9, v/v). EPC: egg phosphatidyl choline; Chol: Cho lesterol.

Establishment of hepatoma model

One milliliter of suspension containing 10^7 Walker-256 (W256) carcinosarcoma cells (Shanghai Medical Industrial Institution) was injected into the thigh muscle of a carrier rat (not included in experimental rats). One week after inoculation, a palpable tumor was found at the injection site. Viable tumor tissue was excised under sterile conditions and soaked in 20 mL of Hanks balanced salt solution. Tissue was cut into approximately 1 mm × 1 mm × 1 mm fragments. Experimental rats were anesthetized with intramuscular injection of Sumianxin at 0.2 mL/kg. Median incision beneath the metasternum was made and the liver was exposed. The tumor fragment was implanted into the left liver lobe.

Administration through hepatic artery

On the 7th d after tumor implantation, all animals received laparotomy again. The longest (a) and shortest (b) diameters of the tumor were measured. The tumor volume was calculated as

$$\frac{a \times b^2}{2}$$

By cannulation method described previously[8], normal saline (NS), FADM, LADM, or ADM mixed with blank liposomes (ADM+BL) were injected into the hepatic artery of rats in groups 1-4 respectively. The dose of ADM in each formulation was 2.0 mg/kg body weight. The concentration of ADM was 1.0 mg/mL.

Assessment of therapeutic effect

Tumor growth inhibition: Seven days later, all rats received the third laparotomy. The longest (a) and shortest (b) diameters of the tumor were measured again and the tumor volume after drug administration was calculated. The tumor volume ratio (TVR) was calculated as

$$TVR = \frac{\text{Tumor volume after administration}}{\text{Tumor volume before administration}}$$

Tumor necrosis degree: Seven random rats in each group were killed and anatomized. Hepatoma was removed completely and fixed in 10% formalin. Three 5 μm thick sections from each tumor were sliced on the maximal transverse plane and mounted on glass slides overnight at room temperature. After HE staining, the sections were examined under microscope. According to the percentage of necrosis area, tumors were graded according to the following criteria: Ⅰ, 0%-30%; Ⅱ, 30%-70%; Ⅲ, 70%-100%.

Increased life span: Survival time of the remaining 8 rats in each group was recorded. The mean survival time of NS group was reckoned as control. Increased life span (% ILS) was calculated as

$$\%ILS = \left(\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1\right) \times 100\%$$

Statistical analysis

SPSS 10.0 for windows was used for statistical analysis. Statistical significance was tested by ANOVA and Dunnett test for tumor growth inhibition, log rank test for survival time. $P < 0.05$ was considered statistically significant.

RESULTS

Tumor growth inhibition

No difference in tumor volume was found among the four groups before treatment ($P > 0.05$, Table 2). After treatment, the tumor grew rapidly in rats which received NS. The mean tumor volume was 31.55 times greater than that before treatment. Metastases were observed in about half of these rats. In contrast, the rate of tumor growth lowered apparently in rats which received FADM or ADM + BL ($P < 0.01$). No difference between FADM and ADM + BL was observed ($P > 0.05$). The slowest tumor growth was found in rats which received ia administration of LADM. The mean tumor volume ratio on the 7th day after LADM infusion was 1.243. Statistics indicated that LADM produced a further significant tumor inhibition, as compared with FADM or ADM + BL ($P < 0.01$). In addition, no metastasis was found in rats receiving LADM.

Tumor necrosis degree

Under microscope, W256 tumor cells of rats that received NS showed extensive proliferation and active mitoses. Small areas of necroses were observed in the center of tumor tissue accompanied with a few inflammatory cells (Table 3). Moderate to severe necroses were found in tumors of rats that received FADM or ADM+BL. Grade Ⅲ of tumor necroses was found in 4 of 7 tumors after administration of LADM, including 2 cases of complete tumor necrosis.

Table 2 Tumor volume (V) and tumor volume ratio (TVR) after treatment (mean ± SD)

Group	V (cm^3)		TVR
	Before treatment	After treatment	
NS	0.086 ± 0.049	2.521 ± 0.840	31.550 ± 7.975
FADM	0.083 ± 0.035	0.149 ± 0.072	1.883 ± 0.708[b]
ADM + BL	0.079 ± 0.036	0.136 ± 0.069	1.847 ± 0.661[b]
LADM	0.084 ± 0.049	0.105 ± 0.062	1.243 ± 0.523[d]

[b] $P < 0.01$ *vs* NS; [d] $P < 0.01$ *vs* FADM, ADM + BL.

Table 3 Tumor necrosis degree after treatment

Grade	NS	FADM	ADM + BL	LADM
Ⅰ	6	2	1	0
Ⅱ	1	3	4	3
Ⅲ	0	2	2	4[1]

[1] Complete necrosis in 2 cases.

Table 4 Mean survival time and increased life span after treatment (mean ± SD)

Group	Mean survival time (d)	% ILS
NS	11.88 ± 2.80	-
FADM	20.75 ± 6.34	74.66[b]
ADM + BL	23.13 ± 7.75	94.70[b]
LADM	39.38 ± 11.15	231.48[a]

% ILS : Increased life span. [a] $P < 0.05$ *vs* FADM, ADM + BL; [b] $P < 0.01$ *vs* NS.

Increased life span

Compared with the survival time (11.88 d) of control rats, survival time was greatly prolonged in rats that received LADM (mean, 39.38 d), or FADM (mean, 20.75 d), or ADM+BL (mean, 23.13 d) (Table 4). LADM prolonged the life span by 231.48%, which was significantly longer than FADM (74.66%) and ADM + BL (94.70%) ($P < 0.01$).

DISCUSSION

Hepatocellular carcinoma (HCC) is by far the third most common cause of cancer mortality throughout the world, especially in the Asian-Pacific region, and its incidence continues to increase. On the other hand, HCC has a high expression rate of multi-drug resistance gene (MDR1) and consequently high levels of P-glycoprotein (P-gp), resulting in its insensitiveness to most chemotherapeutic drugs[9]. Few agents show response rates (RRs) above 20%. ADM is one of the most successful anticancer agents to date for the treatment of HCC. Early phase Ⅱ trials and case series

reported RRs ranging from 25% to 100% with single-agent ADM[10]. However, despite its excellent antitumour activity, ADM has a relatively low therapeutic index, and its clinical utility is frequently limited due to acute and chronic toxicities such as myelosuppression, immunosuppression, and dose-cumulative cardiotoxicity. A retrospective analysis of three phase Ⅲ trials of patients treated with ADM in combination with other cytotoxic agents or radiation therapy demonstrates that ADM-associated cardiac events may occur more frequently and at lower cumulative doses than previously reported[11]. Encapsulation of ADM into liposomes may minimize the side effects and enhance the antitumor efficacy by altering its pharmacokinetics and biodistribution pattern. Growing evidence supports the use of LADM as a substitute for ADM to increase the therapeutic index by maintaining the antitumor efficacy while improving the safety profile[12,13]. After intravenous (iv) administration, LADM is predominantly uptaken by the reticuloendothelial system (RES), especially by the liver and spleen[14].

The results of our experiments demonstrated that the antitumor activity of ADM could be markedly enhanced when the drug was encapsulated in liposomes and administered into the hepatic artery. Equivalent or similar effects could not be obtained using FADM or ADM + BL. Compared with FADM or ADM + BL, LADM produced a more significant tumor inhibition and more extensive tumor necrosis. The average tumor volume on the 7th day after treatment with LADM was 0.105 cm^3, about 70% of 0.149 cm^3 of the volume after treatment with FADM. The survival time of rats that received LADM was evidently increased. Compared with saline controls, LADM prolonged the life span by 231.48%, which was significantly longer than FADM (74.66%) and ADM + BL (94.70%).

The likely explanations for the improved therapeutic activity of LADM are as follows. First, W256 carcinosarcoma proliferated rapidly with abundant vasculogenesis, which strongly increased the uptake of LADM by tumor tissues after ia administration. In addition, the deformity of tumor vessels caused significant retention of LADM in tumor tissues. Secondly, it has been testified that the cytocidal effect of ADM depends on the concentration and duration of exposure[1]. Increasing ADM concentration in tumor cells or slowing its elimination could certainly enhance its antitumor efficacy, both of which could be met after ia administration of LADM. Thirdly, encapsulating the drug into liposomes might modify its distribution pattern in tissues. After being administered into rats, liposomes were taken up selectively by RES, such as the liver, spleen, and bone marrow[14]. Accumulation of biodegradable liposomes with associated drugs in Kupffer cells would create a gradient of drug concentration for a massive and prolonged diffusion of the free drug towards neoplastic tissues. Fourthly, the tiny size of the liposome could increase its contact areas significantly, which might result in an apparent improvement in its bioavailability.

We did not include iv administration in the present experiment because the ia route is much more efficient than iv in the treatment of liver malignancies. Different from the liver parenchyma, which receives more than 70% of its blood supply from the portal vein and the rest from the hepatic artery, hepatoma receives approximately 90% of its blood supply from the hepatic artery. The uniqueness of liver blood supply determines the major difference between the two routes of administration. The difference does not warrant repeat comparison in our study.

Our experiments support the hypothesis that the therapeutic effect of TAC could be dramatically enhanced by combination with nanotechnology. We conclude that liposomes can be used as a promising drug carrier in TAC for the treatment of liver primary and metastatic tumors.

REFERENCES

1 **Rupniak HT**, Whelan RD, Hill BT. Concentration and time-dependent inter-relationships for antitumour drug cytotoxicities against tumour cells in vitro. *Int J Cancer* 1983; **32**: 7-12

2 **Kim CK,** Lim SJ. Recent progress in drug delivery systems for anticancer agents. *Arch Pharm Res* 2002; **25:** 229-239

3 **Oussoren C,** Storm G. Liposomes to target the lymphatics by subcutaneous administration. *Adv Drug Deliv Rev* 2001; **50:** 143-156

4 **Ridge JA**, Collin C, Bading JR, Hancock C, Conti PS, Daly JM, Raaf JH. Increased adriamycin levels in hepatic implants of rabbit Vx-2 carcinoma from regional infusion. *Cancer Res* 1988; **48**: 4584-4587

5 **Eksborg S**, Cedermark BJ, Strandler HS. Intrahepatic and intravenous administration of adriamycin--a comparative pharmacokinetic study in patients with malignant liver tumours. *Med Oncol Tumor Pharmacother* 1985; **2**: 47-54

6 **Hope MJ,** Nayar R, Mayer LD, Cullis PR. Reduction of liposome size and preparation of unilamellar vesicles by extrusion techniques. In: *Liposome Technology*. Vol I. 1st ed. Gregoriadis ed. 1993: 123-131

7 **Cullis PR,** Hope MJ, Bally MB, Madden TD, Mayer LD, Fenske DB. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. *Biochim Biophys Acta* 1997; **1331:** 187-211

8 **Zou YY**, Ueno M, Yamagishi M, Horikoshi I, Yamashita I, Tazawa K, Gu XQ. Targeting behavior of hepatic artery injected temperature sensitive liposomal adriamycin on tumor-bearing rats. *Sel Cancer Ther* 1990; **6**: 119-127

9 **Huang CC**, Wu MC, Xu GW, Li DZ, Cheng H, Tu ZX, Jiang HQ, Gu JR. Overexpression of the MDR1 gene and P-glycoprotein in human hepatocellular carcinoma. *J Natl Cancer Inst* 1992; **84:** 262-264

10 **Nowak AK,** Chow PK, Findlay M. Systemic therapy for advanced hepatocellular carcinoma: a review. *Eur J Cancer* 2004; **40:** 1474-1484

11 **Swain SM,** Whaley FS, Ewer MS. Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. *Cancer* 2003; **97:** 2869-2879

12 **Schmidinger M,** Wenzel C, Locker GJ, Muehlbacher F, Steininger R, Gnant M, Crevenna R, Budinsky AC. Pilot study with pegylated liposomal doxorubicin for advanced or unresectable hepatocellular carcinoma. *Br J Cancer* 2001; **85:** 1850-1852

13 **Safra T**. Cardiac safety of liposomal anthracyclines. *Oncologist* 2003; **8** Suppl 2: 17-24

14 **Bao A, Goins B, Klipper R**, Negrete G, Phillips WT. Direct ^{99m}Tc labeling of pegylated liposomal doxorubicin (Doxil) for pharmacokinetic and non-invasive imaging studies. *J Pharmacol Exp Ther* 2004; **308:** 419-425

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RAPID COMMUNICATION

Role of detection of microsatellite instability in Chinese with hereditary nonpolyposis colorectal cancer or ordinary hereditary colorectal cancer

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Abstract

AIM: To detect microsatellite instability (MSI) in patients with hereditary nonpolyposis colorectal cancer or ordinary hereditary colorectal cancer and to provide criteria for screening the kindreds with hereditary nonpolyposis colorectal cancer at molecular level.

METHODS: MSI was detected in the specimens from 20 cases with HNPCC, 20 cases with ordinary hereditary colorectal cancer and 20 cases with sporadic colorectal cancer by means of polymerase chain reaction-single strand conformation polymorphism.

RESULTS: The positive rate of MSI was 85% (17/20) in HNPCC group, 40% (8/20) in ordinary hereditary colorectal cancer group and 10% (2/20) in the sporadic colorectal cancer group respectively. The differences were significant. The mean ages of the three groups were 43.6, 52.2, and 61.8 years respectively, which increased gradually. The incidence of right hemicolon cancer was 64.7%, 37.5%, and 0% respectively, which decreased gradually and had significant difference. The expression ratio of BAT26 and BAT25 was 94.1% respectively, which was highest in the 5 gene sites studied. The incidence of poorly differentiated adenocarcinoma was 70.6% in HNPCC group among high frequency microsatellite instability (MSI-H), which was higher than the other two groups, which had 50% and 50% respectively.

CONCLUSION: The incidence of MSI-H is higher in HNPCC group. The detection of MSI is simple and economical and has high correlation with the clinicopathologic feature of HNPCC and can be used as a screening method to detect the germ line mutation of the mismatch repair gene.

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Key words: Hereditary nonpolyposis colorectal cancer; Microsatellite instability; Ordinary hereditary colorectal cancer; Single strand conformation polymorphism; Polymerase chain reaction

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant inheritance syndrome, with a penetrance as high as 70%-80%[1], and accounts for about 5%-15% of all colorectal cancer[2] . The molecular genetic basis of the disease is germ line mutation of the mismatch repair (MMR) gene, which causes failure of the DNA MMR system to repair errors that occur during the replication of DNA and results in alterations in the length of simple, repetitive microsatellite sequences and so called microsatellite instability (MSI). MSI may reflect the mutation of the MMR gene indirectly and can be used as a means of screening gene mutation of the MMR gene[3,4]. Recently, studies showed most patients with HNPCC have MSI[5,6] and the ratio is higher than that of patients with sporadic colorectal cancer[7,8]. In the current study, we tested microsatellites of the former paraffin-embedded tissue by the method of polymerase chain reaction-single strand conformation polymorphism(PCR-SSCP) of the Chinese patients who fulfilled the criteria for HNPCC and ordinary hereditary colorectal cancer and tested its application value in the clinic.

MATERIALS AND METHODS

Patients

HNPCC group (group A): 20 patients (12 men, 8 women, mean age 48 years, range 32-70 years) who fulfilled the criteria for HNPCC of Chinese people[9] were selected and their family histories were obtained by follow-

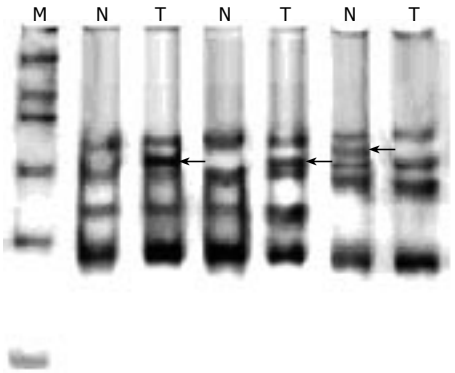

Figure 1 Positive results of MSI. (arrowhead indicates positive results). M: normal DNA; N: normal tissue; T: cancer tissue.

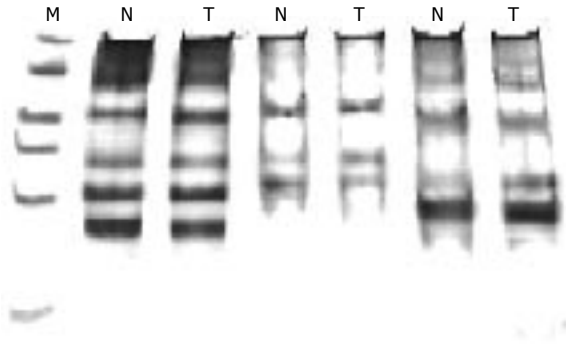

Figure 2 Negative results of MSI (results of 3 pairs are all negative). M: normal DNA; N: normal tissue; T: cancer tissue.

up study. Among them 9 cases were with carcinoma of ascending colon, 2 cases with carcinoma of transverse colon, 1 case with carcinoma of descending colon, 2 cases with carcinoma of sigmoid colon and 6 cases with carcinoma of rectum.

Ordinary hereditary colorectal cancer group (group B): 20 patients (13 men, 7 women, mean age 61 years, range 30-83 years) who fulfilled the criteria for ordinary hereditary colorectal cancer of Chinese people[10] were selected. Among them 5 cases were with carcinoma of ascending colon, 3 cases with carcinoma of transverse colon, 1 case with carcinoma of descending colon, 2 cases with carcinoma of sigmoid colon and 9 cases with carcinoma of rectum.

Sporadic colorectal cancer group (group C): 20 patients (10 men, 10 women, mean age 65 years, range 42-80 years) who were diagnosed with colorectal cancer by pathology and with no family history were selected. Among them 5 cases were with carcinoma of ascending colon, 4 cases with carcinoma of sigmoid colon and 11 cases with carcinoma of rectum.

Methods

For MSI analysis, normal and tumor tissues of the three groups were embedded with paraffin, 4-5 slides of tissue with thickness of 4 μm were sliced and stained with HE. Normal and tumor tissues were selected with microscopy. They were transferred to the EP tubes which contained 150 μL cell lysates. Then DNAs of the normal and tumor tissues were extracted with DNA extraction kit. The primers of the 5 microsatellite loci of HNPCC (BAT26, BAT25, D2S123, D5S346 and D17S250) were selected according to recommendation of the international cooperation organization (Table 1). The primers were synthesized by TAKARA Company.

The reaction system: It was consisted of a total volume of 25 μL with 3 μL template DNA, 0.5 μL forward and reverse primers, 10 × Buffer 2.5 μL, dNTP 2.0 μL, TaqE 0.2 μL, DMSO 1.0 μL, ddH_2O 15.3 μL. The PCR program started with a 95℃ denaturation for 5 min, then at 94℃ for 40 s, 53℃ for 30 s followed by 35 cycles of annealing at 72℃ for 1 min, and extension at 72℃ for 40 s, finally an extension at 72℃ for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide.

Detection of MSI: Single strand conformation polymorphism (SSCP) was used to analyze MSI. The PCR products of normal and tumor tissues were mixed with the same volume of alkaline buffer, then started with 97℃ denaturation for 10 min and put in the mixture of water and ice for 5 min. Then electrophoresis was performed vertically on the 10% nondenaturing polyacrylamide gel (constant power; 60 W) for about 4 h. When the indicating straps reached the inferior margin of the gel or the tracer agent disappeared, silver staining, coloration, fixation and termination were performed. When the film dried 24 h later, the imaging was observed.

Results assessment: MSI was defined by the presence of novel bands following PCR amplication of tumor DNA, which were not present in PCR products of the corresponding normal DNA. If more than 2 of them showing positive results, it was defined as high frequency microsatellite instability (MSI-H), and if only 1 of them showing positive result, it was defined as low frequency microsatellite instability (MSI-L), and if none of them showing positive result, it was named microsatellite stable (MSS).

RESULTS

The results of detection of MSI are shown in Figures 1 and 2. and Tables 1 and 2.

The relationship among MSI-H, mean age of the patients and the sites of cancer are shown in Table 3.

The ratio of every locus occupying in the MSI-H is shown in Table 4.

The clinical pathological characteristics of colorectal cancer are as follows: in the 17 cases with MSI-H of group A, 7 cases had multiple cancers, among which 4 cases were with multiple cancers simultaneously, 3 cases were with multiple cancers at different times. As to groups B and C, none of the patients were with multiple cancers. There were 70.6% (12/17) of poorly differentiated adenocarcinoma in group A, which was more than that of groups B (50%, 4/8) and C (50%, 1/2).

DISCUSSION

Microsatellites are long stretches of apparently redundant DNA between genes by 2-6 nucleotides tandemly arrayed, within which repetitive sequences may be found. Repetition of two bases(CA/GT) is most common. Under normal conditions, the repetitive sequence is constant but

Table 1 Primer sequence of MSI and annealing temperature

Locus	Primer sequence	Annealing temperature (℃)
BAT26	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACCC	53.9
BAT25	TCGCCTCCAAGAATGTAAGT TCTGCATTTTAACTATGGCTC	53.4
D2S123	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	57.4
D5S346	ACTCACTCTAGTGATAAATCGGG AGCAGATAAGACAGTATTACTAGTT	54.0
D17S250	GGAAGAATCAAATAGACAAT GCTGGCCATATATATATTTAAACC	53.6

Table 2 Detection of MSI in three groups (%)

Group	*n*	MSI-H	MSI-L	MSS
A	20	85 (17/20)	10 (2/20)	5 (1/20)
B	20	40 (8/20)	35 (7/20)	25 (5/20)
C	20	10 (2/20)	30 (6/20)	60 (12/20)

MSI-H: high frequency microsatellite instability; MSI-L: low frequency microsatellite instability; MSS: microsatellite stable.

highly polymorphic and is an ideal mark for localization of genes. Cells lacking normal DNA mismatch repair acquire mutations that change the length of nucleotide repeat sequences, termed microsatellite instability (MSI). MSI is the pattern of manifestation of MMR and has a close relationship with HNPCC[11,12], therefore the mutation of MMR results in the incidence of MSI. About 70%-90% of the patients with HNPCC had MSI and only 10%-15% occurred in patients with the sporadic colorectal cancer. Although the Amsterdam Criteria (the International Collaborative Group established in 1991) is now used as the diagnostic criteria for HNPCC clinically, its determination depends mainly on family history and clinical symptoms and cannot reflect the true morbidity of the disease and predict the occurrence of HNPCC. Therefore the detection of mutation of MMR genes has become the golden standard for diagnosis of HNPCC. However, the MMR gene is very large, it has no mutational hot spot, and the cost of detection is expensive, thus it cannot be used as a routine means for screening of HNPCC family at present. As a phenotype of MMR gene mutation, MSI can be used as criterium for detection of HNPCC, especially in patients whose family history is not complete and who do not fit the criteria of HNPCC clinically. Therefore, DNA is extracted from the tumor tissue for detection of MSI. When a patient of MSI-H is diagnosed, detection of MMR gene should be done. As to the choice of loci of the microsatellite instability, 5 loci of microsatellite are used for PCR amplification according to the recommendation of the International Collaborative Group and the International Cancer Research Institute and the Collaborative Group of the Chinese Genetic Colorectal Cancer. However, number of the microsatellites detected is not constant, from less than 3 loci to more than 100 loci. The criteria for the number and ratio of the positive locus of MSI tumor are not uniform. Therefore the results of many different researchers could not be compared with each other.

Table 3 Relationship among MSI-H, mean age of the patients and the sites of cancer (%)

Group	*n*	MSI-H	Mean age (yr)	Right hemicolon	Left hemicolon
A	20	17 (85)	43.6	11 (64.7)	6 (35.3)
B	20	8 (40)	52.2	5 (62.5)	3 (37.5)
C	20	2 (10)	61.8	0 (0)	2 (100)

Table 4 Ratio of every locus occupying in the MSI-H (%)

Locus	Positive ratio of MSI-H	Positive ratio of MSI
BAT26	94.1 (16/17)	85 (17/20)
BAT25	94.1 (16/17)	90 (18/20)
D2S123	64.7 (11/17)	55 (11/20)
D5S346	52.9 (9/17)	50 (10/20)
D17S250	35.3 (6/17)	40 (8/20)

In this study, we amplified 5 loci of microsatellite by PCR according to the criteria of the International Collaborative Group and the International Cancer Research Institute and found the positive ratio was as high as 85% for MSI-H patients with HNPCC, which is in accordance with other research. We found the 5 loci were ideal for detection of MSI of HNPCC patients. It reflected the mutation of MMR gene objectively and could be used as a reliable indicator for screening of HNPCC family just before gene sequencing. If other loci were selected, the type of MSI could be decided by the percentage of positive locus, and MSI-H could be diagnosed when the percentage of positive loci was more than 30%-40% while MSI-L was diagnosed when the percentage of positive loci was less than 30%. As to which loci should be selected, there are a variety of viewpoints. Some scholars think 1-2 microsatellite loci are enough. Hoang[13] believes BAT26 has high correlation with MSI, and their concordance rate is very high. Because the detecting process is relatively simple, Stone[14] detected MSI from tumor specimen using BAT26 directly and recommended that it should be used more extensively. Our study found the positive rate of BAT26 was as high as 94.1%, indicating its high sensitivity for MSI-H detection. At the same time, the positive rate of BAT25 was also 94.1%. Their annealing temperature was close to each other and could be carried out in the same reaction system. The combined application of BAT26 and BAT25 may elevate the specificity of the experiment.

Moreover, our research found that about 40% of the patients with ordinary hereditary colorectal cancer were classified as MSI-H, although it was lower than that of the HNPCC group, it was higher than that of the sporadic colorectal cancer group (the positive rate was 10%), suggesting that about 30% of the patients with ordinary hereditary colorectal cancer had the characteristics of HNPCC. Whether these patients were potential HNPCC

patients needs further study. MSI has been shown to have a close relationship with the onset age of the patients in many recent studies. MSI-H shows good consistency with early onset of age. Edmonston[15] found that the patients whose onset of age was less than 41 years were all MSI-H positive, and the youngest onset age of MSI-H was less than 26 years. Terdiman[16] found that in the different HNPCC families, the final diagnosis age of MSI-H was young and there were more family members with HNPCC than other families, and the ratio of multiple cancers or other HNPCC-related cancer increased. Our research revealed that the mean onset age of MSI-H in the HNPCC group was 43.6 years, which was younger than that of the ordinary hereditary colorectal cancer group (52.2 years) and the sporadic colorectal cancer group (61.8 years). The incidence of multiple cancers or other HNPCC-related cancers of HNPCC group was higher than other groups. In addition, MSI had a close relationship with the sites of carcinoma. The carcinoma of MSI-H, either of HNPCC or of sporadic colorectal cancer, was all likely to occur at the proximal half of a colon. Just as Altonen's study showed[17], 62% of the tumors occurred at the proximal hemicolon of the patients with HNPCC while 38% occurred at the distal hemicolon, suggesting that MSI had a close relationship with cancer of right hemicolon and may play a fundamental role in the pathology of cancer of right hemicolon. Our research found the incidences of right hemicolon cancer of the HNPCC group and the ordinary hereditary colorectal cancer group were 64.7% and 62.5% respectively, which were higher than those of left hemicolon cancer. There was no cancer of right hemicolon found in the sporadic colorectal cancer group, because the occurrence of rectum cancer was higher in North China and the bias was related with the cases selected. Our research suggests that MSI had high correlation with the clinical and pathological characteristics of the HNPCC.

At present, there have been few reports about MSI in typical cases of Chinese people and no clinical data about MSI in patients with ordinary hereditary colorectal cancer. Our research demonstrates that the incidence rate of MSI-H in patients with HNPCC, the mean onset age, the site of carcinoma occurrence, and the clinical and pathological characteristics of patients with HNPCC are all different from that of patients with sporadic colorectal cancer. Ordinary hereditary colorectal cancer is a special type between HNPCC and sporadic colorectal cancer. It has partial characteristics of HNPCC but is not the same as HNPCC and has many differences from sporadic colorectal cancer. Whether it has a special molecular genetic mechanism needs further study. In addition, the survival rate of patients with HNPCC is higher than that of patients with sporadic colorectal cancer, which is due to the early diagnosis of some patients with HNPCC[18,19]. Recently, studies reported that MSI induced by abnormal expression of MMR may affect the therapeutic effect of chemotherapy and the prognosis of patients with colorectal cancer[20-22]. Therefore, as an important screening method for HNPCC, MSI has a promising prospect in clinical application.

REFERENCES

1 **Cunningham C**, Dunlop MG. Molecular genetic basis of colorectal cancer susceptibility. *Br J Surg* 1996; **83**: 321-329

2 **Müller A**, Fishel R. Mismatch repair and the hereditary non-polyposis colorectal cancer syndrome (HNPCC). *Cancer Invest* 2002; **20**: 102-109

3 **Liu T**, Wahlberg S, Burek E, Lindblom P, Rubio C, Lindblom A. Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer. *Genes Chromosomes Cancer* 2000; **27**: 17-25

4 **Debniak T**, Kurzawski G, Gorski B, Kladny J, Domagala W, Lubinski J. Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining hMLH1 and hMSH2 gene mutations in patients with colorectal cancer. *Eur J Cancer* 2000; **36**: 49-54

5 **Lynch HT**, Smyrk T. Hereditary nonpolyposis colorectal cancer (Lynch syndrome). An updated review. *Cancer* 1996; **78**: 1149-1167

6 **Liu B**, Nicolaides NC, Markowitz S, Willson JK, Parsons RE, Jen J, Papadopolous N, Peltomäki P, de la Chapelle A, Hamilton SR. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet* 1995; **9**: 48-55

7 **Brentnall TA**, Crispin DA, Bronner MP, Cherian SP, Hueffed M, Rabinovitch PS, Rubin CE, Haggitt RC, Boland CR. Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res* 1996; **56**: 1237-1240

8 **Aaltonen LA**, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin JP, Järvinen H, Powell SM, Jen J, Hamilton SR. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993; **260**: 812-816

9 The Collaborative Group of the Chinese Genetic Colorectal Cancer. The practice project of the screening standard for Chinese Genetic Colorectal Cancer. *Zhonghua Zhongliu Zazhi* 2004; **26**: 191-192

10 **Liu WZ**, Jin F, Wang SB. The Analysis of Clincal Phenotype of the Herediatery Nonpolyposis Colorectal Cancer and the Common Herediatery Colorectal Cancer. *Zhongguo Zhongliu Linchuang* 2005; **32**: 811-813

11 **Bocker T**, Diermann J, Friedl W, Gebert J, Holinski-Feder E, Karner-Hanusch J, von Knebel-Doeberitz M, Koelble K, Moeslein G, Schackert HK, Wirtz HC, Fishel R, Rüschoff J. Microsatellite instability analysis: a multicenter study for reliability and quality control. *Cancer Res* 1997; **57**: 4739-4743

12 **Boardman LA**. Heritable colorectal cancer syndromes: recognition and preventive management. *Gastroenterol Clin North Am* 2002; **31**: 1107-1131

13 **Hoang JM**, Cottu PH, Thuille B, Salmon RJ, Thomas G, Hamelin R. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Res* 1997; **57**: 300-303

14 **Stone JG**, Tomlinson IP, Houlston RS. Optimising methods for determining RER status in colorectal cancers. *Cancer Lett* 2000; **149**: 15-20

15 **Edmonston TB**, Cuesta KH, Burkholder S, Barusevicius A, Rose D, Kovatich AJ, Boman B, Fry R, Fishel R, Palazzo JP. Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. *Hum Pathol* 2000; **31**: 1506-1514

16 **Terdiman JP**, Gum JR Jr, Conrad PG, Miller GA, Weinberg V, Crawley SC, Levin TR, Reeves C, Schmitt A, Hepburn M, Sleisenger MH, Kim YS. Efficient detection of hereditary nonpolyposis colorectal cancer gene carriers by screening for tumor microsatellite instability before germline genetic testing. *Gastroenterology* 2001; **120**: 21-30

17 **Aaltonen LA**, Peltomäki P, Mecklin JP, Järvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res*

1994; **54**: 1645-1648
18 **Lynch HT**, Lynch J. Lynch syndrome: genetics, natural history, genetic counseling, and prevention. *J Clin Oncol* 2000; **18**: 19S-31S
19 **Lynch HT**, Lynch JF. Hereditary nonpolyposis colorectal cancer. *Semin Surg Oncol* 2000; **18**: 305-313
20 **Gryfe R**, Kim H, Hsieh ET, Aronson MD, Holowaty EJ, Bull SB, Redston M, Gallinger S. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000; **342**: 69-77
21 **Cawkwell L**, Li D, Lewis FA, Martin I, Dixon MF, Quirke P. Microsatellite instability in colorectal cancer: improved assessment using fluorescent polymerase chain reaction. *Gastroenterology* 1995; **109**: 465-471
22 **Hemminki A**, Mecklin JP, Järvinen H, Aaltonen LA, Joensuu H. Microsatellite instability is a favorable prognostic indicator in patients with colorectal cancer receiving chemotherapy. *Gastroenterology* 2000; **119**: 921-928

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RAPID COMMUNICATION

Pharmacodynamic and kinetic effect of rabeprazole on serum gastrin level in relation to CYP2C19 polymorphism in Chinese Hans

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Abstract

AIM: To observe the pharmacokinetics and pharmacodynamics of rabeprazole and compare serum gastrin concentrations in different CYP2C19 genotype groups.

METHODS: The CYP2C19 genotype status of Chinese Han healthy volunteers was determined by polymerase chain reaction-restriction fragment length polymorphism method. Twenty *H pylori*-negative healthy subjects voluntary participated in the study. They were divided into the following three groups: homozygous extensive metabolizers (homEM), heterozygous extensive metabolizers (hetEM) and poor metabolizers (PM). After they orally received rabeprazole 20 mg once daily in the morning of d 1 and d 8, blood samples were collected at various time-points until 24 h after administration and intragastric pH values were monitored for 24 h by Digitrapper pH. Serum gastrin concentrations were measured by radioimmunoassay. Serum concentrations of rabeprazole were measured by high performance liquid chromatography.

RESULTS: The mean AUC values for rabeprazole after a single and repeated doses were significantly different between the homEM and PM groups, but not between the homEM and hetEM, or the hetEM and PM groups. No significant differences in intragastric pH medians were observed among the three different genotype groups after a single dose or repeated doses. The ratio of pH medians between d 1 and d 8 ranged from 84% to 108%. The mean gastrin AUC values were also different among the three genotype groups, with a relative ratio of 1.0, 1.2 and 1.5 after a single dose and 1.0, 1.5 and 1.6 after repeated doses in the homEM, hetEM and PM groups, respectively. The gastrin AUC values among the three different genotype groups showed no significant difference either after a single dose or repeated doses. The subject who had lower intragastric acidity showed higher serum gastrin levels and concentrations of rabeprazole.

CONCLUSION: In Chinese Han healthy people, the pharmacokinetics of rabeprazole are dependent on the CYP2C19 genotype status, but acid-inhibitory efficacy of rabeprazole and the gastrin level are not influenced significantly.

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Key words: CYP450; Pharmacokinetics; Pharmacodynamics; Proton pump inhibitors

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INTRODUCTION

CYP450 2C19 is a genetically determined enzyme. Its phenotypes are either poor metabolizer (PM) or extensive metabolizer (EM)[1,2] in the general population. When CYP2C19 is the main metabolism enzyme of a drug, the pharmacokinetics and pharmacodynamics of the drug are different between PMs and EMs. Rabeprazole (RPZ), a kind of the newer proton pump inhibitors (PPIs), has been reported to be metabolized mainly *via* a non-enzymatic pathway, with only minor CYP2C19 and CYP3A4 involvement. The pharmacokinetics of RPZ are expected to be less influenced by the CYP2C19 phenotype than those of omeprazole[3-6]. However, it is not clear whether the pharmacokinetics and pharmacodynamics of RPZ depend on the CYP2C19 genotype status in Chinese Han people. On the other hand, it is well known that gastrin is secreted from G cells in the antrum of the stomach[7], and an inhibition of gastric acid secretion by PPIs can stimulate gastrin release from G cells[8]. Until now, little is known about the kinetics and pharmacodynamic effect

of RPZ on gastrin levels with respect to the polymorphic CYP2C19 in Chinese Han people. Therefore, in the present study, we observed the metabolic characteristics and pharmacodynamics of RPZ and serum gastrin levels after the single and 8-d repeated doses in the different CYP2C19 genotype groups. It showed that acid-inhibitory efficacy of rabeprazole and the gastrin levels were not influenced significantly by CYP2C19 genotype.

MATERIALS AND METHODS

Subjects and CYP2C19 genotypes

Twenty healthy male subjects who were negative for *H pylori* infection were enrolled in the study. None of the subjects consumed alcohol or smoked, and none had taken any drugs at least 4 wk before or during the study. The protocol was approved in advance by the ethic committee of Anhui Medical University. Written informed consent was obtained from each of the subjects before participation in the study.

Genomic DNA was extracted from leucocytes of each individual using a commercially available kit (Promeg, USA). The genotyping of CYP2C19, including CYP2C19*wt, CYP2C19*m1 and CYP2C19*m2, was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP)[9]. The age of the subjects ranged from 20 to 23 years and body weight from 57 to 63 kg. Subjects were genotypically classified into the following three groups: homozygous extensive metabolizer group (homEM, n = 7), heterozygous extensive metabolizer group (hetEM, Wt/m1, n = 4 ;Wt/m2, n = 2) and poor metabolizer group (PM, m1/m1, n = 7).

Study protocol

Subjects were initially screened for *H pylori* infection by a serological test (Dot-immunogold kit, Lanbo Bio-Tech Institute, China) and ^{13}C-urea breath test. Each healthy volunteer received 20 mg RPZ (Pariet, Eisai Co. Ltd, Tokyo, Japan) orally for 8 d. The medications were taken once daily at 8:00 am. The 24-h intragastric pH monitoring and the measurement of plasma concentration of RPZ and gastrin level were performed on d 1 and d 8. Blood samples were collected before and 0.5, 1, 1.5, 2, 3, 5, 7, 10, 12, 24 h after administration on d 1 and d 8. After collection, the blood samples were immediately centrifuged at 4000 r/min for 10 min. One hundred microliter 1% diethylamine solution was added to the 1 mL plasma sample for determination of the concentration of RPZ. All samples were stored at -80℃ until assayed. Plasma levels of RPZ were measured by high performance liquid chromatography[10,11] . Serum gastrin levels were measured using radioimmunoassay (Gastrin-RIA kit, North Bio-Tech Institute, China).

Intragastric pH measurement

After overnight fasting, a glass electrode was inserted transnasally and placed about 5 cm below the cardia. The electrode was calibrated with standard buffers (pH 1.07 and 7.01) before recording the pH with a Digitrapper pH (Medtronic). Intragastric pH recordings started after the oral dose of RPZ at 8:00 am on d 1 and d 8. Two standard meals (noon, 18:00 pm), prepared at the hospital, were provided for each subject.

Table 1 AUC of RPZ 20 mg after a single and repeated doses

	AUC[a] (μg/L per h) d 1	AUC[a] (μg/L per h) d 8
homEM	1150.24 ± 327.34	1445.28 ± 205.16
hetEM	1539.42 ± 190.29	1640.91 ± 249.51
PM	2015.38 ± 588.88	2495.61 ± 738.61

[a]P < 0.05 homEM *vs* PM.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed in a model-independent manner, and non-compartmental kinetic parameters (AUC) were calculated with 3P87 software. The area under concentration-time curve (AUC) for RPZ and gastrin in serum was shown from zero to 24 h (AUC_{0-24}).

Statistical analysis

The data are given as mean ± SD in all analyses. Differences in AUC and intragastric pH values between three genotype groups were compared using one-way analysis of variance (ANOVA) combined with the least significant method (LSD). To determine whether the gastrin concentrations and AUC values were increased from a single dose to repeated doses, the paired t-test was used. Statistical calculations were performed by SPSS 10.0 software. P value of less than 0.05 was considered to be statistically significant.

RESULTS

CYP2C19 genotype and AUC of RPZ

The mean AUC of RPZ is shown in Table 1. The mean AUC values for RPZ after a single dose differed among the three genotype groups, with a relative ratio of 1.0, 1.3 and 1.8 in the homEM, hetEM and PM groups, respectively. The mean AUC values for RPZ after repeated doses also differed among the three groups, with a relative ratio of 1.0, 1.1 and 1.7 in the homEM, hetEM and PM groups, respectively. The mean AUC values for RPZ after a single and repeated doses were significantly different between the homEM and PM groups, but not between the homEM and hetEM, or hetEM and PM groups. No significant change in the mean AUC values for RPZ from a single dose to repeated doses was observed in any of the three genotype groups.

CYP2C19 genotype and 24-h intragastric pH

The median intragastric pH value of the PM group was the highest, followed by that of the hetEM group, with that of the homEM group being the lowest (Table 2). No significant differences in intragastric pH values were observed among the three groups after a single dose and repeated doses of RPZ for 8 d. No significant changes in intragastric pH values from single to repeated doses were observed in the three genotype groups. The ratio of intragastric pH median after a single dose and that after repeated doses ranged from 84.5% to 107.4%, indicating that the metabolism of RPZ after a single dose could

Table 2 Median (interquartile range) 24 h pH after a single and repeated doses of RPZ 20 mg

	HomEM	HetEM	PM
D 1	3.82 (2.1-5.2)	4.36 (2.0-6.1)	6.09 (4.9-6.8)
D 8	4.52 (3.0-5.5)	4.37 (2.9-6.0)	5.67 (4.8-6.5)
D 1/D 8 (%)	84.5	99.8	107.4

Table 3 Serum gastrin level with reference to CYP2C19 polymorphism

	HomEM		HetEM		PM	
	D 1	D 8	D 1	D 8	D 1	D 8
Gastrin AUC (pg/mL per h)[a]	812.03 ± 147.02	1169.98 ± 333.70	964.08 ± 377.20	1771.38 ± 1024.90	1181.06 ± 420.70	1897.45 ± 1359.59

[a]$P < 0.05$, AUC on d 1 *vs* AUC on d 8.

attain maximum acid-inhibitory efficacy.

CYP2C19 genotype and serum gastrin level

The 24-h mean serum gastrin concentration-time profiles are given in Figure 1. The mean serum gastrin concentration in PMs was significantly higher during all time periods than that in EMs. The mean serum gastrin AUC values in hetEMs observed after a single and repeated doses were between that of homEMs and PMs. The mean gastrin AUC values differed between the three genotype groups, with a relative ratio of 1.0, 1.2 and 1.5 after a single dose and 1.0, 1.5 and 1.6 after repeated doses in the homEM, hetEM and PM groups, respectively (Table 3). The gastrin AUC values among the three genotype groups showed no significant differences after a single dose and repeated doses of RPZ 20 mg. Significant increments in gastrin AUC values from a single dose to repeated doses were observed in the three different genotype groups.

As shown in Tables 1, 2 and 3, the subjects who had the lowest intragastric acidity showed the highest serum gastrin levels and the concentrations of RPZ.

DISCUSSION

PPIs, such as omeprazole and rabeprazole, have been used widely in the treatment of acid-related diseases. Studies have found that CYP2C19 is a major enzyme for the metabolism of PPIs in the liver[12], and the inhibitory effects of PPIs are associated with the genotype of CYP2C19. The *in-vitro* human liver microsomal and *in-vivo* human pharmacology studies have shown that the metabolic profile of RPZ differs somewhat from other PPIs. RPZ is metabolized mainly *via* a nonenzymatic reduction to rabeprazole thioether[12-14], and CYP2C19 and CYP3A4 are partially involved in the metabolism of RPZ[12-14]. In addition, it has been shown that RPZ has a more rapid and powerful onset of pharmacological action[15]. Yasuda[10] found that the AUC for RPZ is not significantly increased after repeated doses. Our results showed that the AUC for RPZ after a single dose exceeded 80 percent of that after

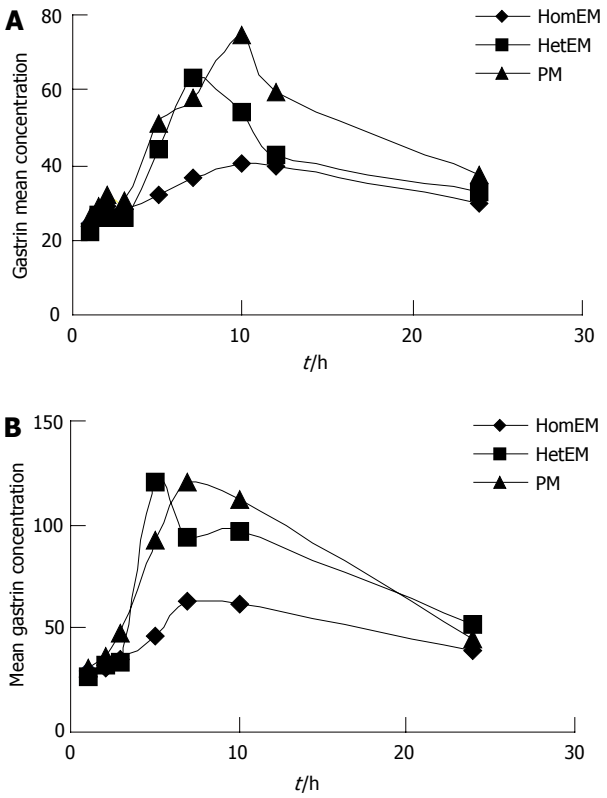

Figure 1 Mean serum gastrin concentration-time curve after a single dose (**A**) and repeated doses (**B**) of rabeprazole 20 mg.

repeated doses, and the gastric pH values after a single dose reached 84.5 to 107.4 percent of those after repeated doses. These findings suggest that the human body could absorb RPZ well, and RPZ could attain better acid-inhibitory efficacy.

Horai[16,17] reported that CYP2C19 genotypic differences affect metabolism and pharmacokinetics of RPZ, and influence the gastric pH values and gastrin level in plasma. However, the majority of researchers[3-6] believe that the acid-inhibitory efficacy and metabolism of RPZ are not dependent on the CYP2C19 genotype status. In our study, the acid-inhibitory efficacy and the gastrin level of rabeprazole were not influenced. This is supposed to be related with the small sample size of our study, as the number of subjects enrolled in each group was small. Ieiri[18] reported that the mean AUC values for rabeprazole differed among the three genotype groups, with a relative ratio of 1.0, 1.7 and 3.8 in the homEM, hetEM and PM groups, being significantly related with genotype status. However, we found the AUC for RPZ was different markedly only between homEMs and PMs. Moreover, the intragastric pH, the best or most direct pharmacological index when using PPIs, had no significant differences among the three genotype groups after a single dose. After repeated doses of RPZ, the intragastric pH and AUC for RPZ were not affected by the CYP2C19 genotype status either. As for the discrepancy between the pharmacokinetics and pharmacodynamics of RPZ, we hypothesized that the acid inhibitory effect of RPZ was powerful and rapid, even in the homEM group.

When a PPI inhibits acid secretion, plasma or serum gastrin levels will be increased according to the degree

of acid inhibition, and serum gastrin concentration correlates well with gastric acid suppression[19]. Therefore, plasma or serum gastrin concentration could be viewed as an indirect marker of the pharmacodynamic effects[20]. Ieiri[18] reported that the intergenotypic difference in the gastrin AUC was seen after the first dose of RPZ, and the AUC of serum gastrin on d 8 differed significantly between homEMs and PMs. However, in our study, no significant difference in the mean gastrin AUC was observed among the three genotype groups on d 1 and d 8, nor were significant differences in the median gastric pH levels found. We presume that the intergenotypic difference in serum gastrin AUC was synchronized with that in median gastric pH, which are consistent with the findings by Furuta[8]. Previous studies indicated that serum gastrin concentrations returned to normal shortly after the antisecretory treatment with RPZ was discontinued[21,22]; however, this phenomenon seems to be in conflict with the longlasting action of PPIs[23]. Therefore, serum gastrin concentrations might not accurately reflect PPI-induced changes in intragastric pH, when PPIs were used in the different CYP2C19 genotype groups.

In conclusion, our study investigated mainly the pharmacodynamic and pharmacokinetic effect of rabeprazole on serum gastrin levels in association with different CYP2C19 genotypes. The acid-inhibitory effects of RPZ are independent of the CYP2C19 genotype status as well as the pharmacokinetic characteristics in the human body. Although the median gastric pH by RPZ treatment is related with the serum gastrin level, there is no significant difference between the different CYP2C19 genotype groups. RPZ is not only an effective proton pump inhibitor for treating the acid-related disease, but also does not affect serum gastrin level in association with CYP2C19 genotype status.

REFERENCES

1 **Andersson T**, Regårdh CG, Dahl-Puustinen ML, Bertilsson L. Slow omeprazole metabolizers are also poor S-mephenytoin hydroxylators. *Ther Drug Monit* 1990; **12**: 415-416

2 **Chiba K**, Kobayashi K, Manabe K, Tani M, Kamataki T, Ishizaki T. Oxidative metabolism of omeprazole in human liver microsomes: cosegregation with S-mephenytoin 4′-hydroxylation. *J Pharmacol Exp Ther* 1993; **266**: 52-59

3 **Adachi K**, Katsube T, Kawamura A, Takashima T, Yuki M, Amano K, Ishihara S, Fukuda R, Watanabe M, Kinoshita Y. CYP2C19 genotype status and intragastric pH during dosing with lansoprazole or rabeprazole. *Aliment Pharmacol Ther* 2000; **14**: 1259-1266

4 **Sakai T**, Aoyama N, Kita T, Sakaeda T, Nishiguchi K, Nishitora Y, Hohda T, Sirasaka D, Tamura T, Tanigawara Y, Kasuga M, Okumura K. CYP2C19 genotype and pharmacokinetics of three proton pump inhibitors in healthy subjects. *Pharm Res* 2001; **18**: 721-727

5 **Hokari K**, Sugiyama T, Kato M, Saito M, Miyagishima T, Kudo M, Nishikawa K, Ishizuka J, Komatsu Y, Mizushima T, Kagaya H, Hige S, Takeda H, Asaka M. Efficacy of triple therapy with rabeprazole for Helicobacter pylori infection and CYP2C19 genetic polymorphism. *Aliment Pharmacol Ther* 2001; **15**: 1479-1484

6 **Miyoshi M**, Mizuno M, Ishiki K, Nagahara Y, Maga T, Torigoe T, Nasu J, Okada H, Yokota K, Oguma K, Tsuji T. A randomized open trial for comparison of proton pump inhibitors, omeprazole versus rabeprazole, in dual therapy for Helicobacter pylori infection in relation to CYP2C19 genetic polymorphism. *J Gastroenterol Hepatol* 2001; **16**: 723-728

7 **Stave R**, Brandtzaeg P. Immunohistochemical investigation of gastrin-producing cells (G cells).The distribution of g cells in resected human stomachs. *Scand J Gastroenterol* 1976; **11**: 705-712

8 **Furuta T**, Ohashi K, Kosuge K, Zhao XJ, Takashima M, Kimura M, Nishimoto M, Hanai H, Kaneko E, Ishizaki T. CYP2C19 genotype status and effect of omeprazole on intragastric pH in humans. *Clin Pharmacol Ther* 1999; **65**: 552-561

9 **de Morais SM**, Goldstein JA, Xie HG, Huang SL, Lu YQ, Xia H, Xiao ZS, Ile N, Zhou HH. Genetic analysis of the S-mephenytoin polymorphism in a Chinese population. *Clin Pharmacol Ther* 1995; **58**: 404-411

10 **Yasuda S**, Ohnishi A, Ogawa T, Tomono Y, Hasegawa J, Nakai H, Shimamura Y, Morishita N. Pharmacokinetic properties of E3810, a new proton pump inhibitor, in healthy male volunteers. *Int J Clin Pharmacol Ther* 1994; **32**: 466-473

11 **Ishizaki T**, Chiba K, Manabe K, Koyama E, Hayashi M, Yasuda S, Horai Y, Tomono Y, Yamato C, Toyoki T. Comparison of the interaction potential of a new proton pump inhibitor, E3810, versus omeprazole with diazepam in extensive and poor metabolizers of S-mephenytoin 4′-hydroxylation. *Clin Pharmacol Ther* 1995; **58**: 155-164

12 **Ishizaki T**, Horai Y. Review article: cytochrome P450 and the metabolism of proton pump inhibitors--emphasis on rabeprazole. *Aliment Pharmacol Ther* 1999; **13** Suppl 3: 27-36

13 **Yasuda S**, Horai Y, Tomono Y, Nakai H, Yamato C, Manabe K, Kobayashi K, Chiba K, Ishizaki T. Comparison of the kinetic disposition and metabolism of E3810, a new proton pump inhibitor, and omeprazole in relation to S-mephenytoin 4′-hydroxylation status. *Clin Pharmacol Ther* 1995; **58**: 143-154

14 **VandenBranden M**, Ring BJ, Binkley SN, Wrighton SA. Interaction of human liver cytochromes P450 in vitro with LY307640, a gastric proton pump inhibitor. *Pharmacogenetics* 1996; **6**: 81-91

15 **Williams MP**, Sercombe J, Hamilton MI, Pounder RE. A placebo-controlled trial to assess the effects of 8 days of dosing with rabeprazole versus omeprazole on 24-h intragastric acidity and plasma gastrin concentrations in young healthy male subjects. *Aliment Pharmacol Ther* 1998; **12**: 1079-1089

16 **Horai Y**, Kimura M, Furuie H, Matsuguma K, Irie S, Koga Y, Nagahama T, Murakami M, Matsui T, Yao T, Urae A, Ishizaki T. Pharmacodynamic effects and kinetic disposition of rabeprazole in relation to CYP2C19 genotypes. *Aliment Pharmacol Ther* 2001; **15**: 793-803

17 **Inaba T**, Mizuno M, Kawai K, Yokota K, Oguma K, Miyoshi M, Take S, Okada H, Tsuji T. Randomized open trial for comparison of proton pump inhibitors in triple therapy for Helicobacter pylori infection in relation to CYP2C19 genotype. *J Gastroenterol Hepatol* 2002; **17**: 748-753

18 **Ieiri I**, Kishimoto Y, Okochi H, Momiyama K, Morita T, Kitano M, Morisawa T, Fukushima Y, Nakagawa K, Hasegawa J, Otsubo K, Ishizaki T. Comparison of the kinetic disposition of and serum gastrin change by lansoprazole versus rabeprazole during an 8-day dosing scheme in relation to CYP2C19 polymorphism. *Eur J Clin Pharmacol* 2001; **57**: 485-492

19 **Robinson M**. Review article: current perspectives on hypergastrinaemia and enterochromaffin-like-cell hyperplasia. *Aliment Pharmacol Ther* 1999; **13** Suppl 5: 5-10

20 **Brand SJ**, Stone D. Reciprocal regulation of antral gastrin and somatostatin gene expression by omeprazole-induced achlorhydria. *J Clin Invest* 1988; **82**: 1059-1066

21 **Lew EA**, Barbuti RC, Kovacs TO, Sytnic B, Humphries TJ, Walsh JH. An ascending single-dose safety and tolerance study of an oral formulation of rabeprazole (E3810). *Aliment Pharmacol Ther* 1998; **12**: 667-672

22 **Breiter J**, Sabesin S, Gardner J, Hahne WF. Rabeprazole relieves heartburn as rapidly as ranitidine and is more effective. *Gastroenterology* 1999; **116**: A128

23 **Thjodleifsson B**, Cockburn I. Review article: rabeprazole's tolerability profile in clinical trials. *Aliment Pharmacol Ther* 1999; **13** Suppl 5: 17-23

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CASE REPORT

Gastric sarcoidosis mimicking irritable bowel syndrome-Cause not association?

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Abstract

Sarcoidosis is a systemic disease of unknown aetiology that may affect any organ in the body. The gastrointestinal tract however is only rarely affected outside the liver. Symptoms may be non-specific. Irritable bowel syndrome (IBS) is a common diagnosis. The recognition of IBS is aided by the use of the Rome II criteria - in the absence of organic disease. We describe the first case of a patient with gastric sarcoidosis who presented with IBS symptoms but subsequently responded to immunosuppressive therapy.

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Key words: Gastrointestinal; Sarcoidosis; Irritable bowel syndrome

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INTRODUCTION

Sarcoidosis is a systemic disease of unknown aetiology characterised by the presence of non-caseating epithelioid granulomas. The lungs, eyes and skin are the most commonly affected organs[1]. Liver involvement is well recognised but symptomatic involvement of the gastrointestinal (GI) tract occurs in less than 1% of cases[2-6]. Presenting GI symptoms in order of frequency are abdominal pain, haematemesis, nausea and vomiting and weight loss[1,2,4-6]. This observation is only based on the case reports and case series that have been reported[2,4-6]. Irritable bowel syndrome (IBS) is a common diagnosis and the symptom based Rome II criteria may be used to support the diagnosis[7]. However it is essential when investigating patients who present with IBS type symptoms that organic disease is excluded[7]. We describe the first case of a patient with gastric sarcoidosis who presented with IBS symptoms but subsequently responded to immunosuppressive therapy.

CASE REPORT

A 27 year old Caucasian man presented to the respiratory physicians with chest discomfort and 'flu-like symptoms in 1983. A plain radiograph of the chest showed bilateral hilar lymphadenopathy and left middle zone infiltrates. Gallium scanning and a positive Kveim test confirmed the diagnosis of pulmonary sarcoidosis. Remission was achieved with oral steroids. Apart from a chronic irritable cough, he had no recurrence of his respiratory symptoms over the following 22 years. His previous annual serum angiotensin converting enzyme (ACE) and a recent high resolution computed tomography (CT) of the chest was normal.

He presented to a gastroenterology department with a 6-mo history of upper/central abdominal pain relieved on defaecation. There was a change in frequency of bowel movement - he now opened his bowels less than three times per week (prior to this presentation he opened his bowels daily). The stool varied between normal and pellet-like. He described bloating, distension and tenesmus. There was no weight loss or any other sinister symptoms. His symptoms fulfilled the Rome II criteria for constipation predominant IBS. His inflammatory markers (ESR and CRP) were normal. Further investigations including an upper GI endoscopy, colonoscopy, ultrasound and CT of the abdomen were all normal. The diagnosis of IBS was suggested but the patient still had concerns about the cause of his symptoms. With the consent of the clinicians from the initial centre the patient sought a second opinion. Although he had no pulmonary symptoms, (apart from a chronic cough) we wondered if his IBS symptoms could be due to gastrointestinal sarcoidosis. Further investigations in our centre showed a raised serum ACE level of 67 IU/mL (Range 18-55 IU/mL). In addition,

Figure 1 Gastric antrum viewed from mid body of stomach showing widespread erythematous changes consistent with gastritis.

Figure 2 Gastro-oesophageal junction showing polypoid lesion.

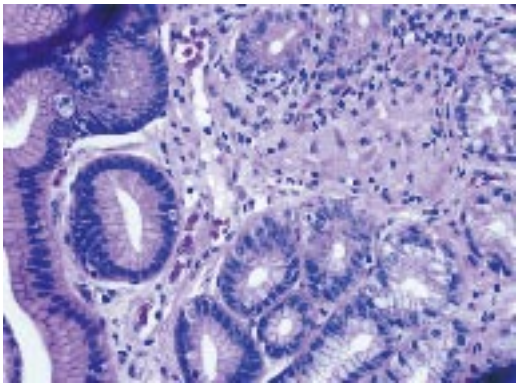

Figure 3 Gastric antral biopsy (HE x 400 magnification) showing surface epithelium on the left of the image. The glands are displaced by a central granuloma formed from loosely aggregated epithelioid histiocytes, with a few surrounding lymphocytes.

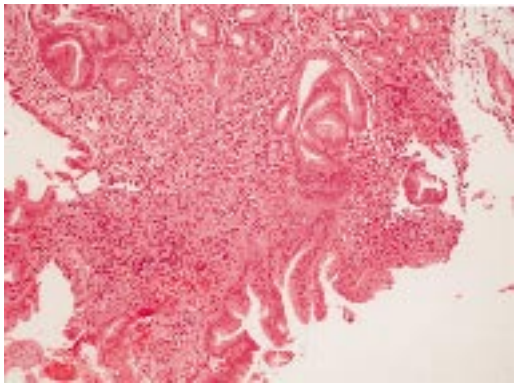

Figure 4 Biopsy of polypoid lesion at gastro-oesophageal junction showing glandular tissue with active chronic inflammation and reactive epithlelial changes (HE x 200 magnification).

he also had a positive IgG antigliadin antibody and a serum IgA of 0.52 G/L (0.8-4.0 G/L) but negative IgA antigliadin and anti-endomysial antibody (EMA). We performed a gastroscopy, which showed antral erythema (Figure 1) and a polypoid lesion at the gastro-oesophageal junction (Figure 2). Duodenal biopsies were normal. Biopsy of the antrum revealed granulomas consistent with sarcoidosis (Figure 3). Biopsy of the polypoid lesion showed chronic inflammatory cell infiltrate only (Figure 4). His CLO test was negative and there was no evidence of *H pylori* or alcohol-acid fast bacilli in any of the biopsy specimens.

Oral prednisolone initially achieved some symptomatic benefit, but he responded subjectively to a short course of intravenous hydrocortisone. During this time his serum ACE normalised and he was discharged on 30 mg prednisolone. He has subsequently had an octreotide scan showing no activity in the stomach (whilst on prednisolone) and was started on oral azathioprine with progressive resolution of his GI symptoms.

DISCUSSION

Sarcoidosis of the GI tract is rare but when involved the stomach is the commonest site. Gastric granulomas have coincidentally been reported in up to 10% of patients with pulmonary sarcoidosis[8,9]. However, whether the presence of gastrointestinal sarcoid accounts for GI symptoms is controversial. Previous investigators have described endoscopic and radiological findings of peptic ulceration, inflammation and gastric outlet obstruction[2,3,5,10-12]. Diagnosis is achieved by biopsy confirming the presence of granulomas without evidence of any other granulomatous disorders. One series of 67 patients with sarcoidosis described 7.5% of patients as having abdominal pains at diagnosis. Further evaluation revealed 15% had liver involvement (biopsy proven) and 36% had luminal GI disease; reflux oesophagitis (21%), IBS (10%) and inflammatory bowel disease (4.5%)[13]. Both reflux and irritable bowel syndrome symptoms are frequent amongst the general population, however, this study suggests that the presence of such symptoms may require investigation. The optimal approach would be upper GI endoscopy and biopsy of any abnormal areas.

This is the first reported case of a patient who fulfilled the Rome II criteria for IBS but was subsequently found to have gastric sarcoidosis. Although we performed antigliadin antibodies (and EMA) to exclude coeliac disease-IgG antigliadin lacks specificity and has previously been reported in association with gastrointestinal sarcoidosis[14,15]. If we had not performed antral biopsies having seen macroscopic evidence of inflammation, then the diagnosis may have been missed. It is possible that in our case both gastric sarcoid and IBS co-exist. We believe that this is unlikely given the symptomatic and serological response to immunosuppression[16,17]. In addition, a normal

octreotide scan whilst on prednisolone (with no areas of increased uptake) also suggests remission and further corroborates our view[16].

In conclusion, we report the first case of gastric sarcoid presenting with Rome Ⅱ criteria for IBS. Our case suggests that patients with suspected functional GI symptoms in the presence of pulmonary sarcoid, may have sarcoidosis of the GI tract. It is imperative that the endoscopist is aware of this possibility and performs a biopsy of any macroscopically abnormal areas. By taking this approach patients with symptomatic GI sarcoid may benefit from early diagnosis and symptomatic relief with prednisolone or immunosuppressive therapy.

REFERENCES

1 **Mitchell D**. Sarcoidosis. *Medicine* 1998; **26**: 43-49
2 **Fireman Z**, Sternberg A, Yarchovsky Y, Abu-Much S, Coscas D, Topilsky M, Fireman E, Groisman GM. Multiple antral ulcers in gastric sarcoid. *J Clin Gastroenterol* 1997; **24**: 97-99
3 **Moretti AM**, Sallustio G, Attimonelli R, Arezzo C, Neri D, Losito A. [Gastric localization of sarcoidosis]. *Recenti Prog Med* 1993; **84**: 750-755
4 **Sprague R**, Harper P, McClain S, Trainer T, Beeken W. Disseminated gastrointestinal sarcoidosis. Case report and review of the literature. *Gastroenterology* 1984; **87**: 421-425
5 **Chinitz MA**, Brandt LJ, Frank MS, Frager D, Sablay L. Symptomatic sarcoidosis of the stomach. *Dig Dis Sci* 1985; **30**: 682-688
6 **Tinker MA**, Viswanathan B, Laufer H, Margolis IB. Acute appendicitis and pernicious anemia as complications of gastrointestinal sarcoidosis. *Am J Gastroenterol* 1984; **79**: 868-872
7 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** Suppl 2: II43-II47
8 **Palmer ED**. Note on silent sarcoidosis of the gastric mucosa. *J Lab Clin Med* 1958; **52**: 231-234
9 **Bratkovskiĭ MV,** Magalif NI. [Sarcoidosis of the stomach]. *Probl Tuberk* 1990;28-30
10 **Farman J**, Ramirez G, Rybak B, Lebwohl O, Semrad C, Rotterdam H. Gastric sarcoidosis. *Abdom Imaging* 1997; **22**: 248-252
11 **Hogg SG**. Case report: gastric sarcoid simulating linitis plastica--a 5-year follow-up study. *Clin Radiol* 1991; **44**: 277-278
12 **Roth D**, West B, Madison J, Cooper D. Gastric carcinoma in a patient with sarcoidosis of the gastrointestinal tract. *Am J Gastroenterol* 1994; **89**: 1589-1591
13 **Reynolds HY**. Sarcoidosis: impact of other illnesses on the presentation and management of multi-organ disease. *Lung* 2002; **180**: 281-299
14 **Sanders DS**, Carter MJ, Hurlstone DP, Pearce A, Ward AM, McAlindon ME, Lobo AJ. Association of adult coeliac disease with irritable bowel syndrome: a case-control study in patients fulfilling ROME II criteria referred to secondary care. *Lancet* 2001; **358**: 1504-1508
15 **McCormick PA**, Feighery C, Dolan C, O'Farrelly C, Kelliher P, Graeme-Cook F, Finch A, Ward K, Fitzgerald MX, O' Donoghue DP. Altered gastrointestinal immune response in sarcoidosis. *Gut* 1988; **29**: 1628-1631
16 **Vanhagen PM**, Krenning EP, Reubi JC, Kwekkeboom DJ, Bakker WH, Mulder AH, Laissue I, Hoogstede HC, Lamberts SW. Somatostatin analogue scintigraphy in granulomatous diseases. *Eur J Nucl Med* 1994; **21**: 497-502
17 **Sachar DB**, Rochester J. The myth of gastrointestinal sarcoidosis: a case of guilt by association. *Inflamm Bowel Dis* 2004; **10**: 441-443

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CASE REPORT

Sustained viral response of a case of acute hepatitis C virus infection via needle-stick injury

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Abstract

A 29-year-old nurse with a hepatitis C virus (HCV) infection caused by needle-stick injury was treated with interferon-beta starting about one year after the onset of acute hepatitis. The patient developed acute hepatitis C with symptoms of general fatigues, jaundice, and ascites 4 wk after the needle-stick injury. When these symptoms were presented, the patient was pregnant by artificial insemination. She hoped to continue her pregnancy. After delivery, biochemical liver enzyme returned to normal levels. Nevertheless, HCV RNA was positive and the pathological finding indicated a progression to chronicity. The genotype was 1b with low viral load. Daily intravenous injection of interferon-beta at the dosage of six million units was started and continued for eight weeks. HCV was eradicated without severe adverse effects. In acute hepatitis C, delaying therapy is considered to reduce the efficacy but interferon-beta therapy is one of the useful treatments for hepatitis C infection in chronic phase.

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Key words: Hepatitis C virus ; Acute hepatitis; Interferon-beta; Needle stick injury

Kogure T, Ueno Y, Kanno N, Fukushima K, Yamagiwa Y, Nagasaki F, Kakazu E, Matsuda Y, Kido O, Nakagome Y, Ninomiya M, Shimosegawa T. Sustained viral response of a case of acute hepatitis C virus infection via needle-stick injury. *World J Gastroenterol* 2006; 12(29): 4757-4760

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INTRODUCTION

Needle-stick injury is a major cause of acute hepatitis C in health care workers[1]. Acute infection of hepatitis C virus (HCV) frequently progresses to chronic hepatitis, which leads to liver cirrhosis and to hepatocellular carcinoma[2]. Preventing the progress to chronicity is necessary, and several treatment strategies of interferon therapies were reported[3,4,5,6]. Early phase treatment is recommended in order to obtain a sustained virological response (SVR)[5]. On the other hand, optimal timing of treatment is controversial because self-limited hepatitis exists in 15%-30% of acute hepatitis C[7]. Several predictive factors of the spontaneous clearance of HCV were reported but these have not been confirmed in larger numbers and well-designed prospective studies. As wait & see strategy to expect the spontaneous viral clearance, it was reported that 8 to 12 wk of delaying therapies obtained more than 90% of SVR rate, however, a delay by one year reduced the rate[7,8]. We report a case of acute hepatitis C infection caused by needle-stick injury just before pregnancy. After delivery, the patient achieved a SVR by treating with interferon-beta in the chronic phase of acute hepatitis C.

CASE REPORT

Patient

The patient was a 29-year old Japanese woman. She was a clinical nurse. She had a missed abortion at the age of 26. She admitted occasional alcohol intake but she did not receive blood transfusions or operations.

Present illness

She encountered a skin puncture on the hand from a needle contaminated with blood from a patient with chronic hepatitis C on September 15th, 2001. She became pregnant through artificial insemination on October 2nd. She felt general fatigue from October 16th. Her attending physician pointed out the abnormal liver function tests, and then she was referred to Sendai City Medical Center at October 19th. On admission, liver function tests indicated elevated aspartate aminotransferase (AST; 549 IU/L, normal 12-30 IU/L) and alanine aminotransferase (ALT; 870 IU/L, normal 8-35 IU/L) activities. Total bilirubin (1.0 mg/dL, normal 0.2-1.2 mg/dL), albumin (39 g/L, normal 42-53 g/L), and prothrombin time (PT; 93%)

Table 1 Laboratory findings at October 19th

WBC	6530 /μL	T-Bil	1.0 mg/dL	HCV-Ab	positive
Hb	13.0 g/μL	D-Bil	0.7 mg/dL	HCV-RNA	> 850 kIU/L
Plt	200 x 10^3 /μL	Total protein	71 g/L	HCV genotype	1b
PT	93%	Albumin	39 g/L	HAV IgM	negative
AST	549 IU/L	BUN	6.4 mg/dL	HBs-Ag	negative
ALT	870 IU/L	Creatinine	0.5 mg/dL	HBs-Ab	negative
LDH	497 IU/L	Na	140 mEq/L	IgM HBc-Ab	negative
ALP	181 IU/L	K	4.0 mEq/L	EBV IgM	negative
γ-GTP	259 IU/L	Cl	106 mEq/L	HSV IgM	negative
ChE	94 IU/l	Glucose	92 mg/dL	CMV IgM	negative

WBC: white blood cell; Hb: hemoglobin; Plt: platelet; PT: prothrombin time; AST: asparate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; γ-GTP: gamma glutamyl transpeptidase; ChE: choline esterase; T-Bil: total bilirubin; D-Bil: direct bilirubin; BUN: blood urea nitrogen; HCV: hepatitis C virus; HAV: hepatitis A virus; HBs-Ag: hepatitis B virus surface antigen; HBc-Ab: hepatitis B virus core antibody; EBV: Epstein-Barr virus; HSV: herpes simplex virus; CMV: cytomegalovirus.

indicated that hepatic functional reserve was maintained (Table 1). Anti-HCV antibody which was negative before the pregnancy, converted to positive, and HCV RNA test revealed high viral load with genotype 1b. Therefore, she was diagnosed with acute hepatitis C. A small amount of ascites was detected on the surface of the liver by ultrasound imaging.

She hoped for continuation of the pregnancy, thus, conservative medical management was performed and liver function tests improved (Figure 1). After that, HCV RNA decreased below detection sensitivity of the routine quantitative method (Cobas Amplicor HCV Monitor test, Roche Diagnostic Systems, Branchburg, NY, USA) although remaining positive with qualitative method (Cobas Amplicor HCV test, Roche). On June 17th, 2002, she delivered safely. Liver function tests were within normal ranges but HCV RNA still remained positive. At this point, spontaneous viral clearance was not expected, thus, she was admitted to our hospital to receive anti-viral therapy on September 9th 2002.

Physical findings at administration

Physical examination revealed: height 158 cm; weight 54 kg; blood pressure 118/64 mmHg; body temperature 36.3℃; and clear consciousness. The bulber conjunctiva was not icteric. No peripheral edema, vascular spider, and flapping tremor were observed.

Laboratory findings

The result demonstrated normal liver function, no evidence of coagulopathy, anemia, or thrombocytopenia. HCV RNA indicated 1.0 kIU/L, which was under detection sensitivity previously.

Pathology

A liver biopsy specimen taken before anti-viral therapy demonstrated chronic hepatitis with mild activity without fibrosis. Swollen Kupffer cells were seen in diastase-periodic acid-Schiff preparations (Figure 2).

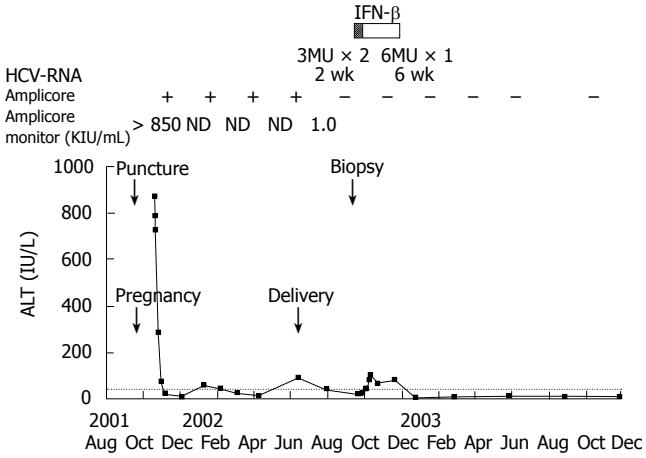

Figure 1 Clinical course of an acute hepatitis C patient receiving interferon-beta (IFN-β). MU, million units.

Clinical course

After informed consent was obtained, the patient received six million units of interferon-beta (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) by intravenous drug injection daily for eight weeks. She developed a daily fever after the initiation of treatment but recovered in a brief period of time. Other adverse effects were not seen during the treatment. HCV RNA became negative two weeks after the initiation of treatment (Figure 1). ALT increased in slight degree but improved after the end of the treatment (Figure 1). HCV RNA negativity and normalization of ALT level were sustained for 40 mo after the end of interferon treatment.

DISCUSSION

In Japan, 85% of hepatocellular carcinoma patients are associated with chronic HCV infection[9]. Thus, to inhibiting the progression of acute HCV infection to chronicity is very important for the prevention of

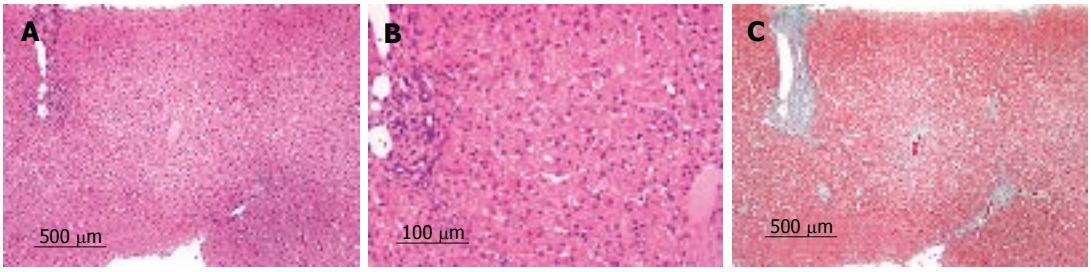

Figure 2 Microscopic pathological findings. **A**: (HE, x 4) and **B**: (HE, x 10) showed inflammatory change with mild activity; **C**: (Elastica-Masson staining, x 4) showed slight fibrotic change.

hepatocellular carcinoma. Recently, as the cause of post-transfusion hepatitis, HCV infection decreased to extremely low levels because of the establishment of diagnostic methods such as HCV-antibody or HCV RNA detection[10]. However, acute hepatitis C still exists by needle-stick injury in health care workers[1]. Interferon therapy has been indicated for acute hepatitis C for many years but the strategies were controversial in the points of optimal timing of starting therapy, treatment regimen, duration of interferon injection, and management of self-limited patients. Therefore the standard therapy for acute hepatitis C has not been established.

Treatment regimen varies with the times and institutes. Interferon-beta was indicated in the early 1990's[11]. Interferon-alpha based therapies have been widely indicated, doses were 3-6 million units, durations were 24-52 wk, and recently, combination with ribavirin and pegylated-interferon were reported[12].

Jaeckel *et al* reported that they treated acute hepatitis C with interferon-alpha (5 million units, thrice weekly for 20 wk) and obtained virological response in 98% of cases, thus, they suggested early treatment immediately on diagnosis[5]. On the other hand, self-limited hepatitis was reported in 15%-30% of acute hepatitis C[7]. identifying the patients who recover spontaneously is important because interferon therapy involves considerable adverse effects, compromises patients' quality of life for long-term treatment, and is highly expensive. Predictable factors of self-limited acute hepatitis C were reported. Female gender[6], younger age[13], and the lack of complications such as human immunodeficiency virus infection[14] were reported as host factors. HCV genotype 3 and 2 were reported as virological factors[15]. Clinical features during the acute phase of infection as symptomatic disease[6] and fast and continuous decline in viral load were reported with a lower rate of chronicity[7]. Hofer *et al* reported that mean duration of spontaneous recovery from acute HCV infection was 77 d from infection and 35 d from the onset of symptoms[7]. Gerlach *et al* reported that they treated acute hepatitis C patients who could not obtain spontaneous viral clearance within 12 wk by interferon-alpha based therapy (3-5 million units, thrice weekly for 14-61 wk) and that the overall virological response was 91% of cases[6]. A meta-analysis indicated that delaying therapy by 60 d after the onset of symptoms did not reduce the efficacy of treatment[16]. Wedemeyer *et al* defined the course of acute hepatitis C virus infection as five phases which were: phase A, post-exposure; phase B, asymptomatic viremic patients with normal ALT, two to six weeks post-exposure before the onset of symptoms; phase C, acute hepatitis with ALT elevation and symptoms; phase D, spontaneous recovery or partial tolerization; phase E, chronic hepatitis after more than six months of infection[17]. Nomura *et al* reported that the SVR to 24 wk of interferon-alpha was 100% when therapy was initiated in acute hepatitis phase, but fell to 53% if treatment was applied in chronic phase[8], though it seems a much high rate to be compared with the SVR rate of conventional interferon monotherapy in chronic hepatitis C.

In our case, we initially expected spontaneous recovery because the patient was of female gender, younger age, because she developed symptoms of jaundice and ascites, and because a fast and continuous decline in viral load was observed. However, HCV RNA level remained at 1.0 kIU/L on year after the onset of the infection. Also liver biopsy indicated the progress to chronic hepatitis. The patient desired to complete the treatment in a short period of time because she has recently completed delivery and she wanted to spend the time with her child and to breastfeed the child, thus we treated her with interferon-beta for eight weeks and obtained the SVR.

Interferon-beta therapy is a useful treatments for acute hepatitis C in chronic phase and is superior to interferon-alpha based therapy with regard to treatment duration. Further studies are necessary to clarify the efficacy of this therapy for acute hepatitis C.

REFERENCES

1 **Kiyosawa K**, Sodeyama T, Tanaka E, Nakano Y, Furuta S, Nishioka K, Purcell RH, Alter HJ. Hepatitis C in hospital employees with needlestick injuries. *Ann Intern Med* 1991; **115**: 367-369

2 **Kiyosawa K**, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671-675

3 **Giuberti T**, Marin MG, Ferrari C, Marchelli S, Schianchi C, Degli Antoni AM, Pizzocolo G, Fiaccadori F. Hepatitis C virus viremia following clinical resolution of acute hepatitis C. *J Hepatol* 1994; **20**: 666-671

4 **Vogel W**, Graziadei I, Umlauft F, Datz C, Hackl F, Allinger S, Grünewald K, Patsch J. High-dose interferon-alpha2b treatment prevents chronicity in acute hepatitis C: a pilot study. *Dig Dis Sci* 1996; **41**: 81S-85S

5 **Jaeckel E**, Cornberg M, Wedemeyer H, Santantonio T, Mayer J, Zankel M, Pastore G, Dietrich M, Trautwein C, Manns MP. Treatment of acute hepatitis C with interferon alfa-2b. *N Engl J Med* 2001; **345**: 1452-1457

6 **Gerlach JT**, Diepolder HM, Zachoval R, Gruener NH, Jung MC, Ulsenheimer A, Schraut WW, Schirren CA, Waechtler M, Backmund M, Pape GR. Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology* 2003; **125**: 80-88

7 **Hofer H**, Watkins-Riedel T, Janata O, Penner E, Holzmann H, Steindl-Munda P, Gangl A, Ferenci P. Spontaneous viral clearance in patients with acute hepatitis C can be predicted by repeated measurements of serum viral load. *Hepatology* 2003; **37**: 60-64
8 **Nomura H**, Sou S, Tanimoto H, Nagahama T, Kimura Y, Hayashi J, Ishibashi H, Kashiwagi S. Short-term interferon-alfa therapy for acute hepatitis C: a randomized controlled trial. *Hepatology* 2004; **39**: 1213-1219
9 **Kiyosawa K**, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17-S26
10 **Alter HJ**, Houghton M. Clinical Medical Research Award. Hepatitis C virus and eliminating post-transfusion hepatitis. *Nat Med* 2000; **6**: 1082-1086
11 **Omata M**, Yokosuka O, Takano S, Kato N, Hosoda K, Imazeki F, Tada M, Ito Y, Ohto M. Resolution of acute hepatitis C after therapy with natural beta interferon. *Lancet* 1991; **338**: 914-915
12 **Zekry A**, Patel K, McHutchison JG. Treatment of acute hepatitis C infection: more pieces of the puzzle? *J Hepato*l 2005; **42**: 293-296
13 **Kenny-Walsh E**. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. *N Engl J Med* 1999; **340**: 1228-1233
14 **Thomas DL**, Astemborski J, Rai RM, Anania FA, Schaeffer M, Galai N, Nolt K, Nelson KE, Strathdee SA, Johnson L, Laeyendecker O, Boitnott J, Wilson LE, Vlahov D. The natural history of hepatitis C virus infection: host, viral, and environmental factors. *JAMA* 2000; **284**: 450-456
15 **Larghi A**, Zuin M, Crosignani A, Ribero ML, Pipia C, Battezzati PM, Binelli G, Donato F, Zanetti AR, Podda M, Tagger A. Outcome of an outbreak of acute hepatitis C among healthy volunteers participating in pharmacokinetics studies. *Hepatology* 2002; **36**: 993-1000
16 **Licata A**, Di Bona D, Schepis F, Shahied L, Craxí A, Cammà C. When and how to treat acute hepatitis C? *J Hepatol* 2003; **39**: 1056-1062
17 **Wedemeyer H**, Jäckel E, Wiegand J, Cornberg M, Manns MP. Whom? When? How? Another piece of evidence for early treatment of acute hepatitis C. *Hepatology* 2004; **39**: 1201-1203

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CASE REPORT

Amyand's hernia: A case report

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Abstract

The presence of vermiform appendix in inguinal hernia is rare and is known as Amyand's hernia. We report an Amyand's hernia, where the appendix was found in a right inguinal hernia in one male cadaver aged ninety two years.

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Key words: Amyand's hernia; Appendix; Inguinal hernia

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INTRODUCTION

Inguinal hernia may display very unusual sac contents. Ovary, fallopian tube, urinary bladder, incarcerated bladder diverticula, large bowel diverticula with the form of diverticulitis or abscess, Meckel's diverticulum (Littre hernia) or foreign bodies (e.g., fishbones) have been rarely reported[1-3]. The presence of the appendix within an inguinal hernia has been referred to as "Amyand's hernia" to honour Claudius Amyand, surgeon to King George II. Amyand was the first to describe the presence of a perforated appendix within the inguinal hernial sac of an eleven-year old boy and performed a successful transherniotomy appendicectomy in 1735[4,5].

CASE REPORT

In a male cadaver, 92 years old, at the time of death, a right inguinal hernia was recognized during a student descriptive anatomy laboratory. The medical history of the patient included arterial hypertension that had been treated for the past twenty years and the right inguinal hernia (without herniotomy) that had existed for the past ten years. The deep fascia, the external oblique aponeurosis and the spermatic cord were identified, as well as the hernial sac. Upon opening the hernial sac, the vermiform appendix was found free within, without adhesions to the sac (Figure 1, Figure 2, Figure 3). Macroscopically, the vermiform appendix was 8 cm in length with a maximum diameter of 0.9 cm. Histological examination did not reveal any pathological alterations (Figures 4A and B).

DISCUSSION

A hernia is defined as the protrusion of a viscus or part of a viscus through the walls of its containing cavity. The presence of the appendix within an inguinal hernial sac is referred to as "Amyand's hernia" and is an uncommon condition. The incidence of having a normal appendix within the hernial sac varies from 0.5% to 1%, whereas only 0.1% of all cases of appendicitis present in an inguinal hernia, underscoring the rarity of the condition[6,7]. The majority of the reported cases present with the features of an obstructed or strangulated inguinal hernia. Even acute appendicitis or perforation of the appendix within the sac simulates perforation of the intestine within the hernia, and does not have specific symptoms or signs. Due to these facts it is very difficult to reach a clinical diagnosis of Amyand's hernia preoperatively. In fact, the diagnosis is made intraoperatively as the patient undergoes surgical exploration for a complicated inguinal hernia. A preoperative computed tomography scanning of the abdomen could be helpful for diagnosis, but this is not a routine practice after the clinical suspicion of a complicated inguinal hernia[8]. However, one case of a three-month old boy has been reported in which a right-sided sliding appendiceal inguinal hernia was diagnosed preoperatively with sonography[9].

The occurrence of herniated appendices is mostly reported in a right inguinal hernial sac, probably as a consequence of the normal anatomical position of the appendix and also because right sided inguinal hernias

Figure 1 Right inguinal hernial sac.

Figure 2 Inguinal hernia with vermiform appendix at the opening of the sac.

Figure 3 Appendix in inguinal hernia.

are more common than left sided hernias[10]. An extensive literature search revealed three reported cases of left sided Amyand's hernia[11-13]. The occurrence of left sided Amyand's hernia may be associated with the presence of a situs inversus or malrotation as an underlying cause. An abdominal computed tomography scan or X-ray examination should be performed to exclude these entities[14].

Most of the published cases have been reported as appendicitis incarcerated in a hernia. It is difficult to determine whether a primary visceral inflammation, which could be referred to as appendicitis, is the pathological

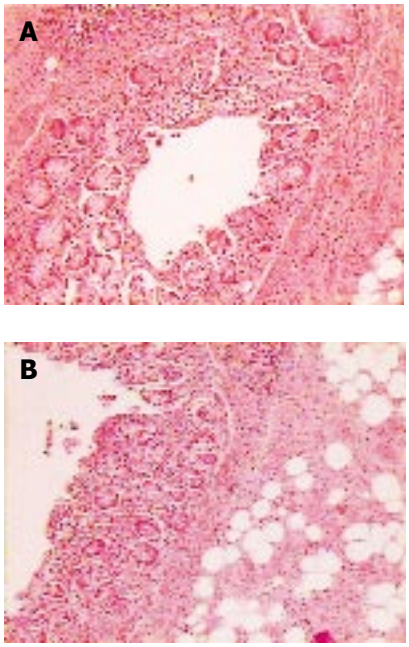

Figure 4 Pathological examination of the appendix found in inguinal hernia. **A**: HE x 100; **B**: HE x 400.

mechanism, or if the primary event is strangulation of the herniated appendix, leading subsequently to ischemic necrosis and secondary inflammation[15]. The presence or absence of inflammation of the appendix is a very important determinant of appropriate treatment. If inflammation of the organ and incipient necrosis are present, a transherniotomy appendectomy should be performed. The presence of pus or perforation of the organ is also an absolute contraindication to the placement of a mesh for hernia repair. Associated intra-abdominal abscesses, if present, may be dealt with either percutaneously or by open drainage. The majority of the authors agree that a normal appendix within the hernial sac does not require appendectomy, and that every effort should be made to preserve the organ found in the hernia sac for an uneventful postoperative course[16,17].

Finally, we conclude that the presence of the appendix in an inguinal hernial sac, referred to as "Amyand's hernia", is an uncommon entity. Despite its rarity, the fact that the majority of such cases present as a complicated inguinal hernia, making preoperative diagnosis difficult, demands that surgeons consider this condition in their differential diagnosis and so they are able to offer appropriate treatment.

REFERENCES

1 **Gurer A**, Ozdogan M, Ozlem N, Yildirim A, Kulacoglu H, Aydin R. Uncommon content in groin hernia sac. *Hernia* 2006; **10**: 152-155
2 **Greenberg J**, Arnell TD. Diverticular abscess presenting as an incarcerated inguinal hernia. *Am Surg* 2005; **71**: 208-209
3 **Casadio G**, Chendi D, Franchella A. Fishbone ingestion: two cases of late presentation as pediatric emergencies. *J Pediatr Surg* 2003; **38**: 1399-1400
4 **Orr KB**. Perforated appendix in an inguinal hernial sac: Amyand's hernia. *Med J Aust* 1993; **159**: 762-763
5 **Hutchinson R**. Amyand's hernia. *J R Soc Med* 1993; **86**: 104-105
6 **Logan MT**, Nottingham JM. Amyand's hernia: a case report of an incarcerated and perforated appendix within an inguinal

hernia and review of the literature. *Am Surg* 2001; **67**: 628-629
7 **Thomas WE**, Vowles KD, Williamson RC. Appendicitis in external herniae. *Ann R Coll Surg Engl* 1982; **64**: 121-122
8 **Luchs JS**, Halpern D, Katz DS. Amyand's hernia: prospective CT diagnosis. *J Comput Assist Tomogr* 2000; **24**: 884-886
9 **Celik A**, Ergün O, Ozbek SS, Dökümcü Z, Balik E. Sliding appendiceal inguinal hernia: preoperative sonographic diagnosis. *J Clin Ultrasound* 2003; **31**: 156-158
10 **Pellegrino JM**, Feldman SD. Case report: acute appendicitis in an inguinal hernia. *N J Med* 1992; **89**: 225-226
11 **Gupta S**, Sharma R, Kaushik R. Left-sided Amyand's hernia. *Singapore Med J* 2005; **46**: 424-425
12 **Breitenstein S**, Eisenbach C, Wille G, Decurtins M. Incarcerated vermiform appendix in a left-sided inguinal hernia. *Hernia* 2005; **9**: 100-102
13 **Carey LC**. Acute appendicitis occurring in hernias: a report of 10 cases. *Surgery* 1967; **61**: 236-238
14 **Bakhshi GD**, Bhandarwar AH, Govila AA. Acute appendicitis in left scrotum. *Indian J Gastroenterol* 2004; **23**: 195
15 **Weir CD**, Doan SJ, Lughlin V, Diamond T. Strangulation of the appendix in a femoral hernia sac. *Ulster Med J* 1994; **63**: 114-115
16 **Bleichrodt RP**, Simmermacher RK, van der Lei B, Schakenraad JM. Expanded polytetrafluoroethylene patch versus polypropylene mesh for the repair of contaminated defects of the abdominal wall. *Surg Gynecol Obstet* 1993; **176**: 18-24
17 **Franko J**, Raftopoulos I, Sulkowski R. A rare variation of Amyand's hernia. *Am J Gastroenterol* 2002; **97**: 2684-2685

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CASE REPORT

Mutations of the *AAAS* gene in an Indian family with Allgrove's syndrome

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Abstract

The triple A or Allgrove's syndrome is an autosomal recessive disorder characterized by the triad of achalasia cardia, alacrima and ACTH resistant adrenocortical insufficiency. Mutations of the Achalasia-Addisonianism-Alacrima-Syndrome (*AAAS*) gene on chromosome 12q13 are associated with this syndrome. We report an Indian family where two siblings were homozygous for a known mutation of the *AAAS* gene and presented with the classical triad of symptoms. The mother and the brother were heterozygous and asymptomatic. The affected siblings had iron deficiency anemia and the younger sister had pes cavus and palmoplantar keratosis. Neurological symptoms were absent in both affected children. Recognition of this syndrome can lead to early treatment of adrenal insuffiency and genetic counselling.

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Key words: Allgrove's syndrome; Triple A syndrome; Autosomal recessive; Genetic mutation; India

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INTRODUCTION

Allgrove's syndrome, also known as the triple A syndrome is characterized by the triad of adrenal insufficiency, achalasia cardia and alacrima[1]. Ever since its first description in 1978, more than 70 cases have been reported in the literature. The condition is associated with impairment of the central, peripheral and autonomic nervous systems, suggesting that it is a result of a mutation in a pleiotropic gene. Other reported abnormalities include palmoplantar hyperkeratosis, short stature, osteoporosis and microcephaly[2-4]. Progression of neurological symptoms has been reported with worsening of peripheral neuropathy, dementia, long tract degeneration, dysarthria and ataxia[5,6].

Pathological studies from esophageal myomectomy specimens of children afflicted with Allgrove's syndrome show fibrosis of the intermuscular plane and absent myenteric ganglia[3]. Neuronal NO synthase has also been found to be deficient and could explain the poor relaxation of the lower esophageal sphincter (LES). Necropsy has been carried out in one of the pairs of original siblings published, which reveals atrophy of the adrenal cortical zona fasciculata and zona reticularis and absence of ganglion cells and nerve fibers in the lower esophagus[1].

This syndrome has been mapped to a novel gene on chromosome 12q 13 near the type II keratin gene cluster, and the gene is designated as the Achalasia-Addisonianism-Alacrima-Syndrome gene (the *AAAS* gene)[7]. There is a great deal of variability of symptoms and severity between patients with the same *AAAS* mutation, implying variable expression of this mutated gene.

This report describes the clinical presentation of two affected siblings with Allgrove's syndrome and analyses the mutations in the immediate family.

CASE REPORT

A seventeen year old young lady presented along with her fifteen year old younger sister with identical symptoms of dysphagia, excessive fatigue and skin pigmentation. The elder sibling had been symptomatic for eight years and her sister for seven years respectively. Both had progressive difficulty in swallowing and regurgitation of food at the time of presentation. They had extreme tiredness and fatigue. The mother had noticed progressive pigmentation in both her daughters. The younger sibling had several episodes of sporadic abdominal pain and vomiting. Their milestones had been normal and they had attained menarche and thelarche normally. Their parents were non-consanguinous and their father had expired following an accident. One elder sister had died of meningitis at nine years of age and on questioning the mother, she mentioned the daughter having similar facial hyperpigmentation. A brother was asymptomatic at 16 years of age. Examination of both patients revealed normal blood pressure and heart rate with no postural changes. Both sisters had facial and palmar hyperpigmentation. The younger sibling had bilateral

Table 1 Summary of clinical features and laboratory investigation in both siblings with Allgrove's syndrome

Variable	Elder sibling	Younger sibling
Dysphagia	Present	Present
Generalized pigmentation	Present	Present
Abdominal pain	Absent	Present
Decreased lacrimation	Present	Present
Postural hypotension	Absent	Absent
Palmoplantar keratosis	Absent	Present
Temporal optical pallor	Present	Present
Hemoglobin (mg%)	6.8	8.1
Albumin (mg%)	4.2	4.2
Pre-Synacthen cortisol (μg/deciliter)	3.94	3.37
Post-Synacthen cortisol (μg/deciliter)	5.16	4.1
Barium swallow	Aperistaltic dilated esophagus	Aperistaltic dilated esophagus
LES pressure on manometry (mmHg)	45	60

palmo-plantar keratosis and pes cavus. The conjunctiva and oral cavities of both sisters were dry. Schirmer's test confirmed decreased lacrimation. Both were noted to have bilateral temporal pallor of optic discs.

Hemogram showed iron deficiency anemia in both sisters. Biochemical tests and chest X ray were normal. Barium swallow showed dilated esophagus and absent peristalsis in both sisters. Esophageal manometry done to confirm the diagnosis showed elevated LES pressures (45 mm and 60 mm respectively), poor relaxation to swallow and absent body peristalsis consistent with achalasia. Serum 8 am cortisol was 3.94 μg/deciliter and 3.37 μg/ deciliter in both sisters respectively and post Synacthen values were 5.16 μg/ deciliter and 4.10 μg/ deciliter respectively, consistent with Addison's disease. The clinical features and laboratory investigations are summarized in Table 1.

The confirmation of achalasia, Addison's disease, history of dry eye in both siblings, palmo-plantar hyperkeratosis in one sister and positive family history pointed to a clinical diagnosis of Allgrove's syndrome. Molecular testing for the *AAAS* gene causing Allgrove's syndrome was initiated to confirm the diagnosis. Blood samples from the patients, their mother and unaffected brother were taken after consent for *AAAS* gene analysis. Replacement therapy with corticosteroids and mineralocorticoids dramatically improved the symptoms in both the sisters. They have been advised to make frequent follow-up visits at the clinic.

MUTATION ANALYSIS

DNA was extracted using standard protocols. Coding sequences including exon-intron boundaries were amplified from genomic DNA and sequenced as reported previously [8]. Sequencing of the coding regions and exon/intron junctions of the *AAAS* gene in the two sisters revealed a homozygous splice mutation in exon 1, resulting in a frameshift after glycine at amino acid position 14 and a premature stopcodon (G14fs). This is the result of the transversion C to A at nucleotide position 43, forming a new premature splice donor site after G at nucleotide position 40 with a consecutive loss of the following 83 bp from exon 1. The mother is a heterozygote and so is the brother. Thus it verified the carrier status of the unaffected family members. This gene mutation analysis confirmed the diagnosis and helped in genetic counselling.

DISCUSSION

Triple A syndrome (Allgrove's syndrome) is an autosomal recessive genetic disorder occurring due to mutations in the *AAAS* gene, resulting in phenotypic manifestations of achalasia, addisonianism, alacrima and variable symptoms of progressive neurological involvement. Using positional cloning, the product of the *AAAS* gene, designated ALADIN for alacrima, achalasia, adrenal insufficiency neurological disorder, was identified as a protein belonging to the WD repeat family[9]. Proteins belonging to this family have a wide functional diversity in that they are involved in signal transduction, RNA processing, vesicular trafficking, cytoskeleton assembly and cell division control[10].

The ubiquitous expression of *AAAS* is in accordance with the multisystemic character of the disorder and the predominant occurrence in the adrenal gland, gastrointestinal and nervous structures is in line with the tissues mainly affected in triple A syndrome. Various mutations have been described in patients from European, North African, Asian and American populations[8]. Ours is the first report of mutations in the *AAAS* gene in an Indian family with Allgrove's syndrome. The transversion of C to A at nucleotide position 43 results in a markedly truncated protein, and it has been reported and confirmed that the mutation caused the disease[8].

Genotypic-phenotypic correlations have not been consistent in Allgrove's syndrome[11]. We noted similarities in presentation in both these siblings. However, palmoplantar keratosis and pes cavus was present in only the younger sister despite both siblings having the same mutation. There are reports of patients presenting without hypoadrenalism, which is the hallmark of this syndrome[12]. From a large series of patients it has been seen that frameshift, stop codon and functionally significant mutations are likely to lead to a more severe phenotype, most probably occurring by a loss-of-function effect of the mutated protein[13]. Future studies need to focus on functional analysis of this mutated gene in order to comprehend the myriad presentations of the Allgrove's syndrome.

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REFERENCES

1 **Allgrove J**, Clayden GS, Grant DB, Macaulay JC. Familial glucocorticoid deficiency with achalasia of the cardia and deficient tear production. *Lancet* 1978; **1**: 1284-1286

2 **García-Compeán D,** Ramón Martínez H, Villegas-González MJ, Montes J, García Quintanilla F, González JA. [Achalasia,

alacrima without adrenal insufficiency with peripheral and autonomic neurological dysfunction (Allgrove's syndrome)]. *Rev Gastroenterol Mex* 1998; **63**: 33-36

3 **Khelif K**, De Laet MH, Chaouachi B, Segers V, Vanderwinden JM. Achalasia of the cardia in Allgrove's (triple A) syndrome: histopathologic study of 10 cases. *Am J Surg Pathol* 2003; **27**: 667-672

4 **Jacob A**, Parameswaran K, Kishore A. Two siblings with Allgrove's syndrome and extrapyramidal features. *Neurol India* 2003; **51**: 257-259

5 **Grant DB**, Dunger DB, Smith I, Hyland K. Familial glucocorticoid deficiency with achalasia of the cardia associated with mixed neuropathy, long-tract degeneration and mild dementia. *Eur J Pediatr* 1992; **151**: 85-89

6 **Grant DB**, Barnes ND, Dumic M, Ginalska-Malinowska M, Milla PJ, von Petrykowski W, Rowlatt RJ, Steendijk R, Wales JH, Werder E. Neurological and adrenal dysfunction in the adrenal insufficiency/alacrima/achalasia (3A) syndrome. *Arch Dis Child* 1993; **68**: 779-782

7 **Huebner A**, Yoon SJ, Ozkinay F, Hilscher C, Lee H, Clark AJ, Handschug K. Triple A syndrome--clinical aspects and molecular genetics. *Endocr Res* 2000; **26**: 751-759

8 **Huebner A**, Kaindl AM, Knobeloch KP, Petzold H, Mann P, Koehler K. The triple A syndrome is due to mutations in ALADIN, a novel member of the nuclear pore complex. *Endocr Res* 2004; **30**: 891-899

9 **Tullio-Pelet A**, Salomon R, Hadj-Rabia S, Mugnier C, de Laet MH, Chaouachi B, Bakiri F, Brottier P, Cattolico L, Penet C, Bégeot M, Naville D, Nicolino M, Chaussain JL, Weissenbach J, Munnich A, Lyonnet S. Mutant WD-repeat protein in triple-A syndrome. *Nat Genet* 2000; **26**: 332-335

10 **Handschug K**, Sperling S, Yoon SJ, Hennig S, Clark AJ, Huebner A. Triple A syndrome is caused by mutations in AAAS, a new WD-repeat protein gene. *Hum Mol Genet* 2001; **10**: 283-290

11 **Prpic I**, Huebner A, Persic M, Handschug K, Pavletic M. Triple A syndrome: genotype-phenotype assessment. *Clin Genet* 2003; **63**: 415-417

12 **Kimber J**, McLean BN, Prevett M, Hammans SR. Allgrove or 4 "A" syndrome: an autosomal recessive syndrome causing multisystem neurological disease. *J Neurol Neurosurg Psychiatry* 2003; **74**: 654-657

13 **Houlden H**, Smith S, De Carvalho M, Blake J, Mathias C, Wood NW, Reilly MM. Clinical and genetic characterization of families with triple A (Allgrove) syndrome. *Brain* 2002; **125**: 2681-2690

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LETTERS TO THE EDITOR

TREM-1 expression during major abdominal surgery: Comment on the Gonzalez-Roldan *et al* paper

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TO THE EDITOR

I read with a great interest the paper by Gonzalez-Roldan *et al*[1]. The authors reported on the pattern expression of TREM-1 during sepsis and major abdominal surgery as compared to healthy controls and concluded that TREM-1 expression increased on the surface of monocytes after surgery. Several points deserve consideration. First, no data related to TREM-1 expression on neutrophils is provided. Second, of the 7 surgical patients, only 4 had both pre- and post-surgery cytometry analysis. Among these 4 patients, 2 displayed a decrease of TREM-1 expression after surgery. Therefore, it seems quite hazardous, based on these data, to conclude that TREM-1 increases after major uncomplicated surgery, reflecting a systemic inflammatory response.

The other question that arose from this study is related to the determination of the TREM-1 splice variant (svTREM-1). First, it is still unknown whether this variant is translated or not[2]. Anyway, the soluble form of TREM-1 is not believed to be related to this variant[3]. The authors observed an increase of the *svTREM-1* mRNA after surgery or during sepsis. By using the described primers, one could also expect to see a PCR product corresponding to the natural form of TREM-1[3]. Unfortunately, such a product is not reported. Finally, it is rather disappointing not to see any measurement of plasma sTREM-1 in these patients in order to better characterise the "inflammatory" patients and the septic ones[4].

REFERENCES

1 **González-Roldán N,** Ferat-Osorio E, Aduna-Vicente R, Wong-Baeza I, Esquivel-Callejas N, Astudillo-de la Vega H, Sánchez-Fernández P, Arriaga-Pizano L, Villasís-Keever MA, López-Macías C, Isibasi A. Expression of triggering receptor on myeloid cell 1 and histocompatibility complex molecules in sepsis and major abdominal surgery. *World J Gastroenterol* 2005; **11**: 7473-7479

2 **Gingras MC**, Lapillonne H, Margolin JF. TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. *Mol Immunol* 2002; **38**: 817-824

3 **Gibot S**, Kolopp-Sarda MN, Béné MC, Bollaert PE, Lozniewski A, Mory F, Levy B, Faure GC. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 2004; **200**: 1419-1426

4 **Gibot S**, Cravoisy A, Levy B, Bene MC, Faure G, Bollaert PE. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med* 2004; **350**: 451-458

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Meetings

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First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March 2007
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<http://www.congre.co.jp/1st-aphpba>/

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
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International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

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10th World Congress of the International Society for Diseases of the Esophagus
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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