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EDITORIAL

Medical treatment of cholestatic liver diseases: From pathobiology to pharmacological targets

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Abstract

Bile secretion is dependent on the coordinated functions of a number of hepatobiliary transport systems. Cholestasis may be caused by an impairment of bile secretion, an obstruction of bile flow or a combination of the two. The common consequence of all forms of cholestasis is retention of bile acids and other potentially toxic compounds in the hepatocytes leading to apoptosis or necrosis of hepatocytes and eventually to chronic cholestatic liver disease. In certain cholestatic disorders there is also leakage of bile acids into the peribiliary space causing portal inflammation and fibrosis. The following pharmacological targets for treatment of intrahepatic cholestasis can be identified: stimulation of orthograde biliary secretion and retrograde secretion of bile acids and other toxic cholephils into the systemic circulation for excretion *via* the kidneys to reduce their retention in the hepatocytes; stimulation of the metabolism of hydrophobic bile acids and other toxic compounds to more hydrophilic, less toxic metabolites; protection of injured cholangiocytes against toxic effects of bile; inhibition of apoptosis caused by elevated levels of cytotoxic bile acids; inhibition of fibrosis caused by leakage of bile acids into the peribiliary space. The clinical results of ursodeoxcholic acid therapy of primary biliary cirrhosis may be regarded as the first success of this strategy.

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Key words: Bile secretion; Biliary transport; Cholestasis; Nuclear receptors; Cholestatic liver disease; Primary biliary cirrhosis; Ursodeoxycholic acid

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INTRODUCTION

Great progress has been made in the last decade in our understanding of the molecular basis of bile formation and the pathobiology of cholestasis[1-3]. Targets for medical therapy of cholestasis have been identified which help to understand the established treatments and facilitate the development of new drugs for cholestatic liver disease. In this short review, present concepts of bile formation and cholestasis are briefly summarized and medical treatment of cholestatic liver diseases is illustrated using primary biliary cirrhosis (PBC), the model disease for chronic cholestatic liver disease, as an example.

MOLECULAR MECHANISMS OF BILE FORMATION

Hepatocellular bile is formed by active transport of solutes into the bile canaliculi. Thereby, a local osmotic gradient is established between canalicular bile and sinusoidal plasma. This causes a flow of water, electrolytes and small solutes into the bile canaliculi, mainly *via* a paracellular pathway through the tight junctions which exhibit perm selectivity, and are impermeable for large and negatively charged solutes[4].

The most important driving force for hepatocellular bile formation is the secretion of bile acids from the sinusoidal blood into the bile[3]. Conjugated bile acids, which represent the major fraction of bile acids in the blood, are transported across the basolateral membrane of hepatocytes together with sodium by the sodium-taurocholate cotransporter (NTCP, *SLC10A1*). Unconjugated bile acids and a large variety of other organic anions including bilirubin are taken up by the hepatocytes *via* the organic anion-transporting polypeptide 2 (OATP2, *SLC21A6*). The rate limiting step for bile formation is the active transport of bile acids and other solutes across the canalicular membrane of hepatocytes. This concentrative step is driven by a number of ATP-dependent export pumps (ATP-binding-cassette-transport proteins also known as ABC-transporters). Bile salts are transported by the bile salt export pump (BSEP, *ABCB11*), whereas bilirubin diglucuronide, glutathione, divalent bile acids conjugates and a large variety of other conjugated organic anions are transported by the multidrug resistance associated protein 2 (MRP2, *ABCC2*)[2].

A special ABC-transporter, namely the multidrug resistance P-glycoprotein 3 (MDR3, ABCB4), flipps

phospholipids from the inner to the outer leaflet of the canalicular membrane. This flippase provides phosphotidylcholine for bile which forms mixed micelles with bile acids and cholesterol[2].

The formation and final composition of bile depends on additional transporters in the canalicular membrane of hepatocytes as well as transporters in cholangiocytes which add cholangiocellular bile to hepatocellular bile. Among those, the chloride-bicarbonate anion exchanger 2 (AE2, *SLC10A2*) is present in the apical membrane of both hepatocytes and bile duct epithelial cells, whereas the cystic fibrosis transmembrane conductance regulator (CFTR, *ABCC7*), a chloride channel, is located in the apical membrane of bile duct epithelial cells only[2].

The basolateral membrane of hepatocytes possesses a number of transporters which are expressed during cholestasis and transport solutes in a retrograde fashion back into the blood (see below). These are MRP4 (*ABCC4*) which transports bile acids together with glutathione[5], MRP3 (*ABCC3*) which transports conjugated bilirubin and other organic anions[2,6] and OSTα/OSTβ, a heteromeric organic solute transporter which transports bile acids[7]. During cholestasis, MRP3[8] and OSTα/OSTβ are also upregulated in the basolateral membrane of cholangiocytes[9].

MOLECULAR MECHANISMS OF CHOLESTASIS

Cholestasis can be defined as an impairment of bile flow. The consequences are retention of bile acids, bilirubin and other cholephils in the liver and blood and a deficiency of bile acids in the intestine. Various forms of cholestasis can be caused by an impairment of bile secretion, an obstruction of bile flow or a combination of the two (Figure 1).

Impairment of bile secretion can be inborn, for instance in different forms of progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC), or cystic fibrosis, and it also can be acquired by inflammation, toxins, drugs or hormones[8,10].

Inborn defects of bile secretion: If BSEP is defective because of a gene mutation, PFIC2 or BRIC2 [11] can occur. PFIC2 can be identified by immunostaining of BSEP in liver biopsies[12].

Mutations of MRP2 cause the Dubin Johnson syndrome, which is not a complete cholestasis, but a more selective defect of biliary secretion of organic anions such as bilirubin glucuronide. Mutations of MDR3 cause PFIC3 and mutations of CFTR cause cystic fibrosis[2,8].

Acquired impairment of bile secretion: In inflammatory disorders such as sepsis, bacterial infections, viral hepatitis as well as toxin or drug-induced hepatitis, inflammatory cytokines can impair bile secretion. Thus, TNFα and IL-1β down regulate NTCP and BSEP which are responsible for bile acid transport, as well as OATP2 and MRP2 which are responsible for transport of bilirubin and a variety of other organic ions[13,14].

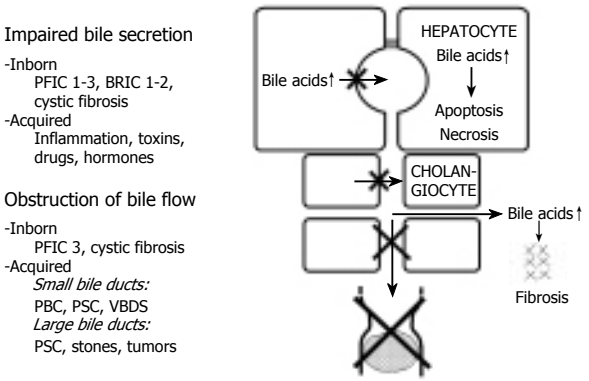

Figure 1 Causes of cholestasis. PBC: Primary biliary cirrhosis; PFIC: Progressive familial intrahepatic cholestasis; PSC: Primary sclerosing cholangitis; VBDS: Vanishing bile duct syndrome. For details see text.

Drugs can cause cholestasis by inhibiting the function of hepatobiliary transport proteins. Some drugs are known to inhibit BSEP directly from the inside of hepatocytes, which is called cis-inhibition. Examples are cyclosporine A, glibenclamide, troglitazone and bosentan[15,16]. Other drugs, such as estradiol 17β-D-glucuronide, must first be transported into the canalicular lumen by MRP2 and then act on BSEP from the luminal side, which is called trans-inhibition[17].

Obstruction of bile flow can also be caused by inborn disorders, e.g. in cystic fibrosis or in PFIC3, and it can be acquired for instance in PBC, primary sclerosing cholangitis (PSC) or the vanishing bile duct syndrome (VBDS). Much more often obstructive cholestasis is caused by stones or tumours.

In cholestatic disorders caused by an initial injury of cholangiocytes (e.g. an immunological injury in the case of PBC), hydrophobic bile acids in bile (in millimolar concentrations) may aggravate the bile duct lesion and contribute to the destruction and loss of bile ducts resulting in progressive obstructive cholestasis. This may be called extracellular bile acid cytotoxicity in contrast to intracellular bile acid toxicity when bile acids accumulate in hepatocytes (in micromolar concentrations). Extracellular bile acid toxicity also occurs towards normal biliary epithelium when phospholipids in bile are low, as in the inborn defect of PFIC3 or in other "low phospholipid syndromes", in low phospholipid gallstone disease[18] or in bile acid phospholipid imbalance in bile after liver transplantation[19].

ADAPTIVE RESPONSES TO CHOLESTASIS

In order to compensate for the loss of biliary excretory function in cholestasis and to limit hepatocellular accumulation of potentially toxic biliary constituents, adaptive responses to cholestasis occur in the liver[6,8,14,20,21], the kidney[20-23] and the intestine[22,24]. In the following only the adaptive changes in the liver are discussed.

Down regulation of NTCP and OATP2 reduces the uptake of bile acids and other organic anions in cholestasis and thus protects the hepatocytes against an overload

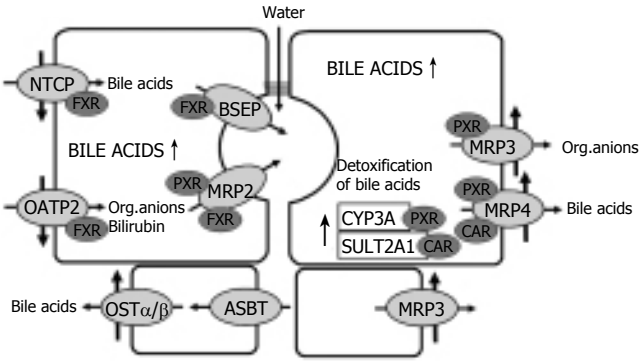

Figure 2 Adaptive responses to cholestasis. BSEP: Bile salt export pump; CAR: Constitutive androstane receptor; CYP3A: Cytochrome P450 enzyme 3A; FXR: Farnesoid X receptor; OATP: Organic anion transporting polypeptide; OST: Organic solute transporter; MRP: Multidrug resistance associated protein; NTCP: Sodium taurocholate co-transporting polypeptide; PXR: Pregnane X receptor; SULT2A1: Sulphotranferase 2A1. For details see tect.

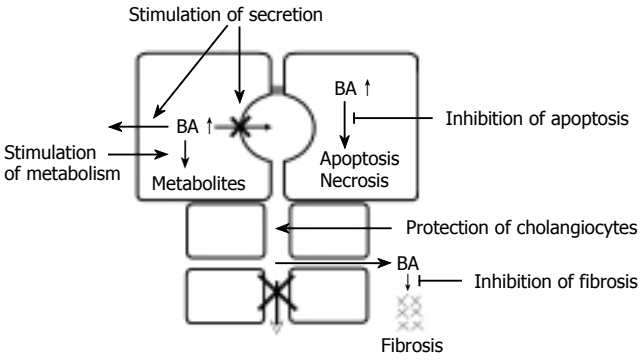

Figure 3 Targets for medical treatment of intrahepatic cholestasis. BA: Bile acids. For details see text.

of bile acids and bilirubin[3,25,26]. At the same time there is upregulation of MRP3 and MRP4 in the basolateral membrane[14,22,27-29]. These transporters normally are expressed at a low level only. MRP4 pumps bile salts and bile salt conjugates together with gluthatione from the cells into the blood and thus decreases bile acid retention in cholestatic hepatocytes. MRP3 mainly exports other organic anions. Prior to their extrusion from hepatocytes, hydrophobic bile acids and many xenobiotics are metabolized to more hydrophilic and less toxic compounds by cytochrome P-450(CYP) 3A enzymes. A large fraction of bile acids is sulphated by the enzyme sulfotransferase 2A1 (Figure 2).

The major players in these adaptive regulations are the nuclear receptors FXR, PXR and CAR[30]. The farnesoid X- receptor (FXR), a bile acid sensor, is mainly involved in the down regulation of NTCP, in the maintenance of BSEP function and in the up-regulation of MRP4 and MDR3. The pregnane X receptor (PXR), to which many xenobiotics bind, is mainly responsible for the up-regulation of MRP3 and various CYP enzymes, especially the family of CYP3A enzymes. There is evidence that more than one of these nuclear receptors can act on the same transporter. Recently, it has been demonstrated that the constitutive androstane receptor (CAR) up-regulates sulfotransferase 2A1 and MRP4 in a coordinated fashion facilitating the conjugation and export of hydrophobic bile acids[31]. In addition to PXR, the perioxisome–proliferator-activated receptor α (PPARα) up-regulates MDR3 (Figure 2).

It is of considerable interest that besides natural bile acids, bile acid derivatives such as ethyl-chenodeoxycholic acid are ligands for FXR[32,33]. Ligands for PXR are many xenobiotics and drugs like rifampicin. Bilirubin and phenobarbital are ligands for CAR and fibrates as well as statins (e.g. pravastatin) bind to PPARα.

These findings open an avenue for the development of drugs which bind to nuclear receptors which enhance normal compensatory mechanisms in cholestasis for the elimination of toxic compounds *via* alternative excretory routes.

TARGETS FOR PHARMACOLOGICAL THERAPY

The common consequence of all forms of cholestasis is retention of bile acids in hepatocytes. Elevated levels of bile acids then can lead to apoptosis or necrosis of hepatocytes and eventually to chronic cholestatic liver disease[34]. In certain cholestatic disorders there is also leakage of bile acids into the peribiliary space, causing portal inflammation and fibrosis *via* induction of chemokines and cytokines[35]. Accordingly, the following pharmacological targets for treatment of intrahepatic cholestasis can be identified (Figure 3): stimulation of orthograde biliary secretion and retrograde secretion of bile acids and other toxic cholephils into the systemic circulation for excretion by the kidneys to reduce their retention in the hepatocytes; stimulation of the metabolism of hydrophobic bile acids and other toxic compounds to more hydrophilic but less toxic metabolites; protection of injured cholangiocytes against toxic effects of bile; inhibition of apoptosis caused by elevated levels of cytotoxic bile acids; inhibition of fibrosis caused by leakage of bile acids into the peribiliary space.

Stimulation of secretion

Secretion of bile acids and other potentially toxic compounds into the bile and blood may be stimulated by enhancing transporter expression and/or function at different levels, namely the levels of transcription, translation, targeting and protein activation.

In mice both cholic acid (CA) and UDCA stimulate the expression of Bsep and Mrp2 mRNA[36]. One must, however, be aware that these findings may not hold true for men, because considerable species differences exist with regard to binding of bile acids to nuclear receptors and regulation of transporter expression by nuclear receptors. Rifampicin, a ligand of PXR, stimulates the expression of MRP2 at the transcriptional level in man[37].

Ursodeoxycholic acid (UDCA) stimulates targeting of the transporters Bsep and Mrp2 to the canalicular membrane in the rat *via* at least two different signalling cascades[38-40]. Immunoelectronmicroscopy with gold particles is employed to assess localization of Bsep in the canalicular membrane and in a subapical compartment of

rat liver. Bsep and Mrp2 in the canalicular membrane are markedly reduced when taurolithocholic acid (TLCA) is administered in the perfused rat liver, but is maintained when tauroursodeoxycholic acid (TUDCA) is added[38,40]. Enhanced expression of BSEP under UDCA treatment in men may contribute to a better elimination of bile acids from the blood. As shown by Poupon *et al*[41], in collaboration with our group, UDCA decreases serum levels of the hydrophobic bile acid, chenodeoxycholic acid (CDCA) in PBC. As shown by Zollner *et al*[14], expression of MRP2 mRNA and protein increases with the enrichment of UDCA in the liver during treatment of patients with PBC and UDCA. Accordingly, as shown by Poupon *et al*[42], UDCA improves excretory function in PBC. Thus, in a randomised, placebo controlled study over two years, in patients with PBC, serum bilirubin was significantly lower in the UDCA group than in the placebo group.

Activation of transporters in the canalicular membrane by UDCA and phosphorylation may also occur[43], which has not yet been sufficiently studied.

Stimulation of metabolism

Stimulation of the metabolism of hydrophobic bile acids produces more hydrophilic and less toxic compounds. Rifampicin, a drug used for the treatment of cholestatic pruritus, stimulates the expression of CYP3A4 mRNA in patients with gallstones. In line with this, Dilger *et al*[44] showed that in patients with early stage PBC, rifampicin stimulates CYP3A metabolic activity as assessed by urinary 6β-hydroxy cortisol, whereas UDCA has no effect.

Protection of cholangiocytes

Protection of cholangiocytes by making the bile more hydrophilic and less toxic appears to be an important therapeutic target. UDCA fulfils this requirement because it renders bile acid composition of bile more hydrophilic and increases biliary phospholipid secretion[45].

Inhibition of apoptosis

Inhibition of apoptosis caused by elevated levels of hydrophobic bile acids[46,47] may also be a therapeutic target in cholestasis. As shown by Rodrigues *et al*[48,49], feeding of the hydrophobic bile acid deoxycholic acid (DCA) to rats increases hepatocyte apoptosis as assessed by the number of tunnel positive hepatocytes. Addition of UDCA inhibits this effect. Toxic bile acids such as CDCA can cause apoptosis of hepatocytes *via* the CD95 receptor with formation of a death inducing signalling complex (DISC) and activation of caspase 8. Caspase 8 then causes mitochondrial membrane permeability transition (MMPT) which leads to activation of effector caspases and apoptosis. In addition, UDCA stabilizes the mitochondrial membrane and inhibits MMPT and apoptosis[48,49]. The antiapoptotic effect of UDCA has also been demonstrated in human hepatocytes[50].

Inhibition of fibrosis

Inhibition of fibrosis may become an important therapeutic target in the future. In the rat with common bile duct ligation, fibrosis can be inhibited by 6-ethyl CDCA (6-ECDCA). The antifibrotic effect of 6-ECDCA appears to be mediated *via* FXR and SHP[33]. Recently, an antifibrotic effect of NOR-UDCA has been described in the Mdr2 knock-out mouse[51]. It remains to be shown whether these findings are relevant to human cholestatic liver diseases, but they point towards a promising new way for the development of drugs to inhibit cholestatic fibrosis.

PHARMACOLOGICAL TREATMENT OF CHRONIC CHOLESTATIC LIVER DISEASES

In the following, primary biliary cirrhosis (PBC), the model disease for chronic cholestatic liver disease, is used as an example for the treatment of chronic cholestatic liver diseases by UDCA. PBC is characterized by an inflammatory lesion of interlobular bile ducts, which results in bile duct destruction and may progress to fibrosis and cirrhosis. Since the etiology of the disease is unknown, presently available therapies aim at inhibiting the underlying pathogenetic processes and delaying the progression of the disease.

The pathogenesis of this slowly progressive disease involves a still unknown immunologic injury of small interlobular bile ducts; aggravation of the bile duct lesion by cytotoxic bile acids; obstruction and loss of small bile ducts followed by cholestasis and retention of bile acids; hepatocyte injury, apoptosis, necrosis, fibrosis and eventually cirrhosis with liver failure.

UDCA, at present, is the only approved drug for PBC. It appears to exert its beneficial effects by rendering bile composition less toxic for the injured biliary epithelium, reducing the retention of bile acids in hepatocytes and inhibiting apoptosis[10,34]. Immunosuppressive agents have met with limited success. They have been found useful in combination with UDCA in selected patients[52,53].

In randomized, double-blind placebo-controlled trials UDCA at doses of 13-15 mg/kg body weight per day could improve serum liver tests including serum bilirubin and other serum markers of cholestasis[42,54-56], the Mayo risk score[56] and liver histology[42,55]. As shown by Pares *et al*[54] and Poupon *et al*[57], UDCA inhibits histological progression in early stage PBC. As shown by Corpechot *et al*[58], UDCA inhibits progression to severe liver fibrosis or cirrhosis in early stage PBC. In line with this is the observation that UDCA delays the onset of esophageal varices[59]. A combined analysis of three of the largest trials showed that treatment with UDCA at doses of 13-15 mg/kg per day for up to 4 years can delay the time of liver transplantation or death[60]. Within the first 2 years of treatment, however, a survival benefit was not seen. Doses lower than 10 mg/kg per day of UDCA are of little benefit in PBC[61]. A meta-analysis of 8 randomized trials which showed no difference between UDCA and placebo in the effects on incidence of death, liver transplantation and death or liver transplantation[62] has a number of shortcomings. In 6 of the 8 studies treatment was evaluated up to 24 mo only and the dose of UDCA was 10 mg/kg per

day or lower in two of the studies. Therefore, improvement of transplant free survival by UDCA as shown in the combined analysis of the three largest studies with doses of 13-15 mg/kg per day and a follow-up of 4 years may not have been detectable in this meta-analysis.

CONCLUSION

Better insight into the pathobiology of cholestasis has provided new concepts for pharmacological therapies of cholestatic liver diseases. Among those, therapy with UDCA has been studied most extensively. In PBC, the model disease for cholestatic liver diseases which has been highlighted in this review, the beneficial effects of UDCA have been documented by randomized controlled trials. Treatment with UDCA appears to be beneficial also in a number of other cholestatic disorders, such as primary sclerosing cholangitis (PSC)[63-65], intrahepatic cholestasis of pregnancy[66,67], liver disease in cystic fibrosis[68-70], progressive familial intrahepatic cholestasis (PFIC)[71] and some forms of drug-induced cholestasis[10].

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EDITORIAL

Therapeutic approaches targeting intestinal microflora in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases, ulcerative colitis, and Crohn's disease, are chronic intestinal disorders of unknown etiology in which in genetically susceptible individuals, the mucosal immune system shows an aberrant response towards commensal bacteria. The gastrointestinal tract has developed ingenious mechanisms to coexist with its autologous microflora, but rapidly responds to invading pathogens and then returns to homeostasis with its commensal bacteria after the pathogenic infection is cleared. In case of disruption of this tightly-regulated homeostasis, chronic intestinal inflammation may be induced. Previous studies showed that some commensal bacteria are detrimental while others have either no influence or have a protective action. In addition, each host has a genetically determined response to detrimental and protective bacterial species. These suggest that therapeutic manipulation of imbalance of microflora can influence health and disease. This review focuses on new insights into the role of commensal bacteria in gut health and disease, and presents recent findings in innate and adaptive immune interactions. Therapeutic approaches to modulate balance of intestinal microflora and their potential mechanisms of action are also discussed.

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Key words: Commensal bacteria; Prebiotics; Probiotics; Innate immunity

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INTRODUCTION

The continuous contact between commensal bacterial flora and the single epithelial cell layer of the mucosal tissue is a characteristic feature of the gastrointestinal system. In particular, interaction between commensal bacteria and mucosal immune system plays an important role in keeping health and disease development. Inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD), are chronic intestinal disorders of unknown etiology in which in genetically susceptible individuals, the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria[1]. The relation between a dysregulated bacterial ecosystem and mucosal inflammation in IBD has been demonstrated in a variety of clinical and basic literatures[2-4]. For example, intestinal lesions of IBD predominate in the distal parts of the gastrointestinal tract where the commensal bacteria are most abundant. The presence of intestinal bacteria is essential for development of experimental colitis in several animal models, such as interleukin (IL)-10 gene knockout (KO) mice[5], T cell receptor α-deficient mice[6] and HLA-B27 transgenic rats[7]. In CD, fecal stream diversion reduces gut inflammation and induces mucosal healing in the excluded intestinal segment, whereas infusion of intestinal contents rapidly induced flare-up of disease[8].

The gastrointestinal tract has developed elaborate mechanisms to coexist with its autologous microflora, but rapidly respond to invading pathogens and then return to homeostasis with its commensal bacteria after the pathogenic infection is cleared. If these tightly regulated homeostatic mechanisms are disturbed, chronic intestinal inflammation may be induced[9]. Previous studies demonstrated that some commensal bacteria are detrimental, and others have either no effect or have a protective action. In addition, each host has a genetically determined response to detrimental and protective bacterial species. Environmental and genetic factors modulate the relative balance of beneficial and detrimental bacterial species, suggesting that therapeutic manipulation of this balance can influence health and disease. This review focuses on new insights into the role of commensal bacteria in gut health and disease, and presents recent advances in therapeutic approaches to modulate imbalance of intestinal microflora in IBD patients.

THE INTESTINAL MICROFLORA IN HEALTH AND DISEASE

The gastrointestinal tract host a complex and dynamic microorganisms enviroment. Most members are from the

domain bacteria, but there are also representatives from archaea and eukarya, as well as virus[10]. The intestinal habitat of an adult human individual contain more than 500 different species of bacteria, with 30-40 species comprising up to 99% of the total population[10]. There is a progressive increase in the number of bacteria along the small bowel, from approximately 10^4 in the jejunum to 10^7 colony-forming units (CFU) per gram of luminal content at the ileal end, with predominance of gram-negative aerobes and some obligate anaerobes[10]. Anaerobes are predominant in the colon, and bacterial counts reach around 10^{12} CFU per gram of luminal content. Bacteria contribute to 60% of the fecal mass. Individuals exhibit variation in the types and numbers of species within their microflora. Conventional culturing techniques could detect only -30% of total bacteria in the gut[11], but the use of molecular biologic techniques enhanced detection capability of numbers and diversity of microflora[4,11].

Intestinal bacteria include native species that permanently colonize the tract and a variable set of microorganisms that transit temporarily through the gastrointestinal tract. Native bacteria are primarily acquired at birth and during the first year, but transient bacteria are being ingested continuously from the external environment. The fetal gut is sterile, and bacterial colonization, which is driven by contact between the infant and its environment, is influenced by the mode of delivery, hygiene levels and medications[4]. At a few days after birth infant feces are rich in enterobacteria species, such as *E coli* and *Bifidobacterium*, and these are soon influenced by feeding habits. Early colonization may also depend on genetic influences[4]. The pattern appear to be determined in part by the host genotype, because similarity in fecal bacterial species is much higher within twins than in genetically unrelated couples who share environment and dietary habits[10]. Intestinal microflora plays an essential role in the development of the gut immune system. Animals bred in a germ-free environment possess architectural abnormalities with crypt hyperplasia and lack of lymphoid follicle development. Immediately after exposure to microbes, the number of mucosal lymphocytes expands in the lamina propria and increases the number of IgA-secreting cells[12,13].

Several studies using different methods have repeatedly demonstrated that the fecal microflora as well as metabolic activity differs between subjects with IBD and healthy controls[14,15]. Recent molecular biology technique revealed that in CD patients the proportion of enterobacteria is increased[16,17], and this finding is compatible with previous reports based in culture techniques[14]. A large part of the dominant microflora (30%) was characterized in undefined phylogenetic groups, indicating a presence of major differences between CD and healthy individuals. Other studies confirmed that *Bacteroides vulgatus* was the only species shared by all CD patients in spite of unusual dominant species. From the results of analyses of mucosa-associated flora, Swidsinski *et al* found high concentrations of mucosal bacteria in patients with bowel inflammation, but not in controls[18]. The concentrations of mucosal bacteria increased progressively with the severity of disease, both in inflamed and non-inflamed colon[18]. They hypothesize that the healthy mucosa is capable of holding back fecal bacteria and that this function is profoundly disturbed in patients with IBD. These observations are compatible with the report by Kleessen *et al*[19]. They demonstrated that more bacteria were detected on the mucosal surface of IBD patients than on those of non-IBD controls[19]. Bacterial invasion of the mucosa was evident in colonic specimens from the UC patients, in the ileal and the colonic specimens from the CD patients, but no bacteria were detected in the tissues of the controls. Colonic UC specimens were colonized by a variety of organisms, such as bacteria belonging to the gamma subdivision of *Proteobacteria*, the *Enterobacteriaceae*, the *Bacteroides/Prevotella* cluster, the *Clostridium histolyticum/Clostridium lituseburense* group, the *Clostridium coccoides/Eubacterium rectale* group, high G + C Gram-positive bacteria, or sulphate-reducing bacteria, while CD samples harbored mainly bacteria belonging to the former three groups. Previously, we also reported that the bacterial counts for both aerobes and anaerobes increased in UC patients[20]. In particular, we detected the highest bacterial counts of *Bacteroides vulgatus* (Table 1). A high agglutination titer against *B. vulgatus, B. fragilis,* and *C. ramosum* was detected in most UC patients, and the percentage of positive immunoreactivity was much higher in UC patients than in healthy controls. The serum immunoreactivity (IgG) against 26-kDa protein derived from *B. vulgatus* outer-membrane was much higher in UC patients (53.8%) than in the control sera (9.1%) (Figure 1). These results suggest that *B. vulgatus* and a specific antibody response directed against it may play an important role in the pathogenesis of UC[20].

Table 1 Comparison of rectal mucosal flora in UC patients

	Bacterial counts (mean ± SD)		Positive rate (%)	
	UC	Control	UC	Control
Total	6.63 ± 0.96	5.47 ± 0.96		
Aerobes	6.23 ± 0.50	5.28 ± 2.47		
Anaerobes	6.42 ± 1.05	5.01 ± 0.76		
Clostridium	4.64 ± 2.07	3.74 ± 2.02	61.1	42.9
Bacteroides	5.33 ± 1.71	3.96 ± 1.66	94.4	85.7
Bifidobacterium	5.32 ± 2.64	4.25 ± 2.24	44.4	28.6
Eubacterium	4.73 ± 2.13	2.73 ± 1.53	33.3	57.1
Fusobacterium	0	4.14 ± 1.95	0	28.6
Actinomyces	2.28 ± 0.83	0	5.5	0
Veillonella	4.82 ± 1.79	0	11.1	0
Peptostreptococcus	4.53 ± 2.17	1.80 ± 1.14	38.8	28.6
Streptococcus	4.93 ± 2.33	3.03 ± 1.47	27.7	14.3
Peptococcus	3.35 ± 1.34	0	11.1	0

$\log_{10}$ numbers of organism/g tissue. Reproduction from reference 20.

MUCOSAL RESPONSE TO LUMINAL BACTERIA

General background

The search for specific pathogens that trigger intestinal inflammation failed to produce conclusive results[21]. Instead, it has been found that reconstitution of germ-free mice with commensal bacteria can be enough to induce IBD in several gene-deficient as well as T cell

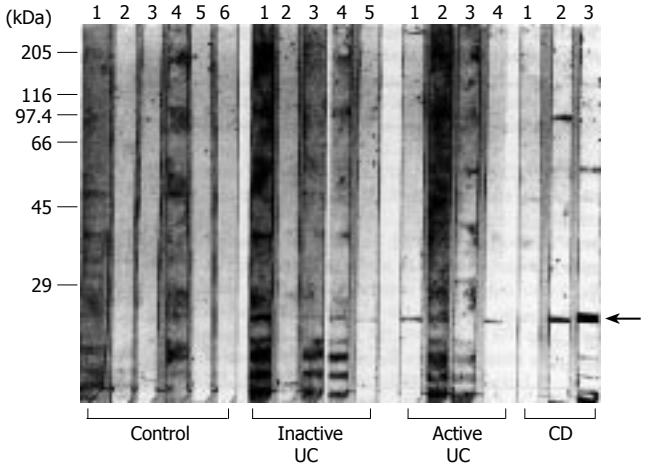

Figure 1 Western blot analysis of serum antibodies (IgG) agaist outer-membrane antigens of Bacteroides vulgatus isolated from an IBD patient. The arrow indicates specific band. Reproduced from reference 20 with permission.

transfer models of IBD in mice[22-25]. Therefore, instead of a specific pathogen, a broad spectrum of bacteria may contribute to the induction of intestinal inflammation. Although metabolically active microbial cells and cell wall components, such as lipopolysaccharide (LPS) and peptidoglycan, present and contact to the host intestinal mucosa, pro-inflammatory responses are absent in the mucosa exposed to the resident luminal microflora. In contrast, the capability to respond to luminal pathogenic bacteria through recruitment of inflammatory cells from systemic circulation is remaining. The probable mechanisms underlying these responses are explained by the standpoints of innate and adaptive immune response[26]. In the intestine, components of innate immunity are preexisting or rapidly activated, resulting in induction or regulation of the highly specific adaptive immune responses.

Recently, novel functions of mucosal dendritic cells (DC) have been reported. Dendritic cells are critical to innate and adaptive immunity as specialized antigen-presenting cells. Hendrik *et al* showed that lamina propria DCs form transepithelial dendrites which enable the cells to directly sample antigens, such as commensal bacterial components[27]. It is likely that DCs take up directly intestinal antigens through transepithelial dendrites and activate an innate immune pathway that protects the mucosa from pathogenic bacteria.

Toll-like receptors and their signaling

Highly conserved structures of pathogenic and commensal bacteria, designated MAMPs (microbe-associated molecular patterns), are recognized by pattern-recognition receptors, such as TLR (Toll-like receptor)[28-30]. TLRs comprise a family of pattern-recognition receptors that detect conserved molecular products of microorganisms, such as LPS and lipoteichoic acid (LTA), recognized by TLR4 and TLR2, respectively. TLRs are expressed both in epithelial cells and in phagocytic cells, thus functioning as sensors of microbial infection. They are critical to initiation of inflammatory and immune defense responses. The bacterial ligands recognized by TLRs are not unique to pathogens, but are rather shared by entire classes of bacteria, and are produced by commensal bacteria as well. However, it remains unclear how the host distinguishes between pathogenic and commensal bacteria. Two major pathways are activated by TLRs[28,30-32]. The first culminate in activating the transcription factor NF-κB, which acts as a master switch for inflammation. It regulates the transcription of many genes that encode proteins involved in immunity and inflammation. The second leads to activation of MAP kinases p38 and Jun amino-terminal kinase (JNK), which also participate in increased transcription and regulate the stability of mRNAs that contain AU repeats. With the exception of TLR3, all TLRs activate NF-κB and MAP kinases *via* a pathway that involves MyD88, IRAK (IL-1 receptor associated kinase)-4 and IRAK-1[30]. There are specific differences in the ultimate gene expression profile that results from the activation of individual TLRs, although the precise mechanisms are unclear. A set of adaptor proteins that are differentially recruited to TLRs may be involved in the molecular basis of this specific gene induction[33].

MyD88 is an adaptor protein for TLRs, and similar to TLRs, it has a Toll-IL-1 receptor (TIR) domain[30-32,34]. Signaling may be initiated by recruitment of MyD88 to TLRs through TIR-TIR interactions. Interaction between MyD88 and TLRs leads to the recruitment of IRAK-4. IRAK-4 becomes activated and is phosphorylated to IRAK-1, resulting in activation of TRAF6[34]. TRAF6 activation results in activation of NF-κB and MAP kinases. Other proteins, such as Tollip, ECSIT, Pellinos, MEKK1 and MEKK3 have also been implicated in this pathway.

Recently, two different groups reported that MyD88-deficient mice reveal an increased susceptibility to dextran sodium sulfate (DSS) colitis[35,36]. MyD88-deficient mice showed an increased mortality and morbidity, as well as aggravation of colitis, following DSS administration. In MyD88-deficient mice, mucosal proliferative zone was expanded and number of proliferating cells increased, indicating dysregulated proliferation and differentiation of intestinal epithelium. Interestingly, similar responses were also observed in TLR2- and TLR4-deficient mice. Increased susceptibility to intestinal injury in MyD88-deficient mice was accompanied with defective production of cytoprotective and reparative factors, such as IL-6, TNF, KC-1 and heat-shock proteins[36]. These findings indicate that TLR signaling *via* the MyD88-dependent pathway conferred protection from the mortality, morbidity and colonic damage caused by the administration of the injurious agent DSS. Since DSS is capable to induce severe morbidity and mortality in wild mice where commensal microflora had been depleted by antibiotics, , Authors have concluded that recognition of commensal microflora by TLRs is required for keeping intestinal homeostasis.

Several mechanisms have been proposed to explain how the epithelium discriminates pathogens from commensals in order to trigger TLR signaling. Although they express similar MAMP, pathogens differ from commensals mainly in their ability to colonize host mucosal surfaces and invade the host. Differences attributed to the differential expression of adhesion molecules. Recently-proposed speculation is that in the gut, the commensal-specific

TLR/MyD88 pathway permits the symbiotic relationship between the microflora and the host, while pathogen-specific virulence factors are required to trigger pro-inflammatory responses *via* the usage of additional TLR co-receptor and/or adaptor molecules for disease-causing organisms[29,37].

NOD2/CARD15

The putative intracellular peptidoglycan receptor NOD2 (CARD15) is a member of the Apaf-1/CARD superfamily and is composed of an N-terminal caspase recruitment domain (CARD), a centrally located nucleotide-binding oligomerization domain (NOD) and 10C-terminal-located leucine-rich repeats (LRRs)[38,39]. NOD2 was found to be expressed in antigen-presenting cells such as monocytes/macrophages, but more recent studies revealed abundant presence of NOD2 in epithelial Paneth cells of the small intestine as well as in other epithelial cells[39,40]. NOD2 has been shown to recognize intracellular peptidoglycan fragments (e.g. muramyl dipeptide, MDP) through its LRR region leading to pro-inflammatory responses through activation of NF-κB. NOD2 serves as an intracellular pattern recognition receptor to enhance host defense by inducing the production of antimicrobial peptides such as human beta-defensin-2[39].

Several studies have shown that mutations in the LRR region of NOD2 are associated with susceptibility to Crohn's disease[41]. The molecular mechanisms by which mutations in the NOD2 gene cause Crohn's disease are still emerging. However, it is supposed that decreased production of antimicrobial peptides, such as defensins, may promote bacterial-mediated inflammation in Crohn's disease[39]. Recent study demonstrated that NOD2 mutation in CD potenciates NF-κB activity and IL-1β processing, suggesting initiation and/or promotion of mucosal inflammation[42].

THERAPEUTIC STRATEGIES TARGETING MICROFLORA IN IBD

IBD continues to be an enigmatic disorder with obvious potential to improve therapeutic target and outcomes[43]. Established therapies for IBD include the aminosalicylates, corticosteroids, and immunosuppressive drugs. An increasing number of novel and alternative therapeutic approaches are in progress[43]. New biologic therapies include the targeting of proinflammatory cytokines, enhancement or infusion of anti-inflammatory cytokines, blocking intravascular adhesion molecules, and modifying T-cell functions. Recently, therapeutic approaches to modifying intestinal microflora have been attempted by using prebiotics and probiotics. In addition, antibiotic therapies continue to be used[2,44-47].

Prebiotics

Prebiotics are nondigestible food constituents that benefit the host by selectively stimulating the growth or activity of one or a limited number of bacterial species already resident in the colon[2]. Some examples of prebiotics are dietary fiber and some types of oligosaccharides. Intake of prebiotics can significantly alter the colonic microflora by increasing the populations of certain bacteria and thereby quantitatively changing the composition of the microflora[48,49]. These alterations may act beneficially, in part, by causing a luminal reduction of short-chain fatty acids (SCFAs), which are both important nutrients for the intestine and inducers of an acidic environment[47,49-52]. Among the SCFAs, butyrate most effectively protects intestinal mucosa against injury and promotes mucosal healing[49,53].

Lactosucrose: Lactosucrose, an indigestible oligosaccharide, is a water-soluble dietary fiber with the potency of modulating microflora. Ohkusa *et al* reported that a daily 6-gram intake of lactosucrose significantly increased the percentage of Bifidobacterium sp and the total number of bacteria in healthy subjects[54,55]. The treatment also reduced fecal ammonia levels but had no effect on fecal SCFA, pH, and water content. It has been reported that an elemental diet or low-fat, low-residual diet decreases anaerobic bacteria and changes the composition of microflora in IBD patients. Teramoto *et al* showed that the continuous administration of lactosucrose for 2 wk led to an increase of Bifidobacterium and a decrease of Bacteroidaceae in patients with IBD[55].

Oligofructose and inulin: Inulin and oligofructose are comparable to dietary fiber in that they are composed of multiple saccharide units, which are indigestible by the enzymes in mammalian small intestine. The saccharide chain in inulin is longer than in oligofructose. Inulin and oligofructose show similar physiological functions in the intestine. It is generally recognized that inulin stimulates the generation of butyrate and the growth of lactic acid bacteria (LAB) in the colons of healthy subjects[50,56]. Videla *et al* examined the efficacy of inulin in a dextran sodium sulfate (DSS)-induced colitis model, demonstrating that it significantly attenuates inflammation as assessed by mucosal damage and both colonic eicosanoid and myeloperoxidase concentrations[50]. The treatment also led to an increase of *Lactobacillus* and a decrease of luminal pH and fecal water content. To our knowledge, no clinical trials to confirm the benefit from either inulin or oligofructose have been performed.

Bran: The laxative effect of wheat bran has long been recognized. Although not potent, bran is used widely because of its harmlessness. A Swedish group examined the effect of wheat bran supplementation on the composition of fecal bile acid and microflora in juvenile patients with UC. Although the clinical activity was not described, wheat bran significantly decreased the number of *Bacteroides* and the concentration of total and unconjugated bile acid[57,58].

Psyllium: Psyllium, also called Ispaghula husk or Plantago ovata, is a water-soluble dietary fiber. Buhman *et al* suggested that psyllium has a hypocholesterolemic effect, based on its hydrocolloid, a gel-forming potency[59,60]. Feeding of psyllium significantly decreases the cholesterol content as well as the cholesterol 7-alpha hydroxylase activity in the rat liver. Hallert *et al* published the first report describing the clinical efficacy of psyllium in patients with UC[61]. Psyllium significantly attenuates clinical symptoms compared with placebo treatment. After this first report was published, a large-scale clinical trial was organized in UC patients by Fernandez-Banares *et al*[62]. In this trial, they

found no significant differences in the remission periods of patients given psyllium treatment and patients given sulfasalazine treatment, although there was an increase in fecal butyrate in the psyllium patients. The authors therefore concluded that psyllium may be as effective as sulfasalazine in maintaining remission in patients with CD.

Germinated barley foodstuff (GBF): GBF, which is derived from the aleuronic layer and scutellum fractions of germinated barley, consists mainly of dietary fiber and glutamine-rich protein[47,48,51,52]. The fiber fraction of GBF consists mainly of low-lignified hemicellulose, which has a large water-holding capacity[63]. GBF contains glutamine, which is another important nutrient for epithelial cells[47,48,52,63]. In germination process, GBF obtains these two unique characteristics of being a glutamine-rich protein and having a conspicuous water holding capacity[64]. In the intestinal lumen, the dietary fiber fraction of GBF is utilized efficiently by *Bifidobacterium* or *Lactobacillus* and converted to lactate and acetate. Coexistence of *Bifidobacterium* and *Eubacterium limosum* increases butyrate production from GBF[65,66]. The endogenous bacterial butyrate produced from GBF is immediately absorbed by the intestinal epithelial cells and utilized by them as an efficient fuel. The water holding capacity of GBF is much higher than other representative water-insoluble dietary fibers, for example, wheat bran, cellulose powder, and sugar beet fiber.

Treatment of rat DSS colitis with GBF in a preventive mode led to a significant improvement of the clinical and pathological signs of colitis and a decrease in serum IL-8 and alpha 1-acid-glycoprotein[64]. The improvements were associated with an induction of luminal butyrate and beneficial organisms, such as *Bifidobacterium* and *Eubacterium*. In a therapeutic mode, GBF was comparably effective against mucosal inflammation and more effective against diarrhea when compared with sulfasalazine[64]. The anti-inflammatory action of GBF was markedly reduced by the concomitant administration of SCFA β-oxidation inhibitor (ibuprofen) and GBF, indicating a butyrate-dependent anti-inflammatory mechanism[67]. In addition to its role as a preferential nutrient for colonocytes, the butyrate also acts as an anti-inflammatory agent by functionally inactivating nuclear factorκB[68]. Of the GBF constituents, the fiber fraction, but not its protein fraction, drastically mitigated mucosal damage with an increase of luminal butyrate. GBF significantly increased the number of *Eubacterium* and *Bifidobacterium*, with a concomitant decrease in luminal pH. In the HLA-B27 transgenic rat, a representative model of spontaneous colitis, GBF improves the clinical and pathological signs of colitis with an increase in luminal butyrate levels[69].

The first trial enrolled 10 patients with mild to moderately active UC who had been unresponsive to or intolerant of standard treatment[70]. The patients consumed 30 g of GBF 3 times daily for 4 wk in a nonrandomized, open-label fashion. At 4 wk, treatment with GBF resulted in clinical and endoscopic improvement with an increase in fecal butyrate. Despite continued treatment with standard drugs, the patients had an exacerbation of the disease within 4 wk after discontinuing GBF treatment. A subsequent multicenter trial with 28 patients conducted in the same fashion showed a similar benefit from GBF. Eighteen patients with mild to moderately active UC were divided into two groups using a random allocation protocol. The control group (n = 7) were given a baseline anti-inflammatory therapy, and the GBF-treated group (n = 11) received 20 to 30 g of GBF daily together with the baseline treatment. After 4 wk of observation, the GBF group showed a significant decrease in clinical activity index scores compared with the control group. No side effects related to GBF were observed[48]. GBF increased fecal concentrations of *Bifidobacterium* and *Eubacterium limosum*. Twenty-one patients with moderately active UC patients were treated with 20-30 g/d of GBF for 24 wk. GBF significantly reduced clinical activity as compared to the control group[52]. Furthermore, GBF prolonged remission in UC patients[51].

Probiotics

In the intestinal lumen of IBD patients, balance between commensal and detrimental bacteria has been broken down with secondary harm on immune system activities. However, the changes in microbiotic composition may be transient; and implantation of exogenous bacteria will have a limited applicability[2,44,46]. Probiotics are live microorganisms administered to alter the intestinal microflora and confer a beneficial effect on health[2]. Potential mechanisms of probiotic action include competitive interactions, production of antimicrobial metabolites, influences on the epithelium, and immune modulation[2,44,46]. However, such changes may be transient, and therefore the implantation of exogenous bacteria has a limited usefulness at present. Restoring the microbial balance using probiotics may be the most physiologic and non-toxic way to prevent and treat IBD.

VSL#3: IL-10 knockout mice develop colitis when they are raised under conventional facilities but not under germ-free conditions. Prior treatment of IL-10 knockout mice with antibiotics prevented the subsequent onset of colitis, suggesting that the exposure to intestinal bacteria during the neonatal period influences later disease progression. The use of VSL#3, a probiotic preparation containing three strains of *Bifidobacterium*, four strains of *Lactobacilli,* and one strain of *Streptococcus salivarius* ssp. *thermophilus* completely normalized the physiological transport function and barrier integrity and also inhibited mucosal TNFα and IFNγ production[71]. *In vitro* studies showed that epithelial barrier function and resistance to Salmonella invasion could be enhanced by exposure to a proteinaceous soluble factor secreted by the bacteria found in the VSL#3 compound[72]. There are increasing number of reports describing anti-inflammatory effects of VSL#3[46].

In a clinical trial, daily administration of VSL#3 prevented relapse of chronic pouchitis after induction of remission by antibiotics. Moreover, every patients relapsed within 3 mo of stopping VSL#3[73]. These were replicated[74,75], and prospective study was performed[76]. In this study, 2 of 20 patients (2%) receiving VSL#3 for one year developed pouchitis versus 40% of placebo-treated patients. Uncontrolled pilot studies have indicated that VSL#3 maintained remission of UC in 75% of patients over 12 mo[73,77]. The results showed that this probiotic preparation could colonize the intestine, and might be useful in maintaining remission in UC. As another study, 32

ambulatory patients with active UC received open label VSL#3, 3600 billion bacteria daily in two divided doses for 6 wk. Treatment of patients with mild to moderate UC, not responding to conventional therapy, with VSL#3 resulted in a combined induction of remission/response rate of 77% with no adverse events. At least some of the bacterial species incorporated in the probiotic product reached the target site in amounts that could be detected[78].

Nissle1917: Previous trials have examined the efficacy of a non-pathogenic strain of Escherichia coli originally called Nissle1917. In the first pilot study, capsules containing this strain of *E coli* were compared with a placebo for the maintenance of prednisolone-induced remission of colonic CD[79]. After 12 wk of treatment, there was no significant difference between the Nissle and control groups. Rembacken *et al* described a single-center trial in which 116 patients with UC were randomized to receive either mesalazine or this non-pathogenic *E coli* strain[80]. Initial responses to treatment were similar, with remission being noted in 75% and 68% of those receiving mesalazine and *E coli,* respectively. Even more impressive were the maintenance benefits; respectively, 73% and 67% of patients remained in remission for 12 mo[80].

***Clostridium butyricum*:** Araki *et al* reported that the anticolitis effect of Japanese microbial preparation (MIYA-IRI-588) was examined in a dextran sulfate sodium (DSS)-induced rodent colitis model[81]. This preparation itself did not display any therapeutic effect. Another probiotic mixture (*Lactobacillus, Clostridium butyricum* and *Streptococcus faecalis*; Biothree: Towa Kasei Co., Ltd, Tokyo, Japan) was also evaluated in a DSS colitis model, by Fukuda *et al*[82]. Although the benefits on colon histology were not significant, diarrhea was significantly decreased by treatment in comparison to the control group 66) Further trials in patients with IBD would be desirable.

Bifidobacterium-fermented milk: The preventive effect of lactic acid bacteria (LAB - *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Lactobacillus acidophilus*)-fermented milk was determined in SAMP1/Yit mice[83]. Administration of LAB-fermented milk to mice reduced histological injury scores, compared with those in saline-treated or unfermented milk-treated mice. Treatment with LAB-fermented milk also reduced ileal tissue weight and myeloperoxidase activity. Moreover, the tissue contents of immunoglobulin such as IgG1 and IgG2a were lower in the inflammatory regions in the LAB-fermented milk-treated group than in the control group. A decreased release of Info and TNFα with an increase of IL-10 from mesenteric lymph node cells were observed in the LAB-fermented milk treated group.

Lactococci-secreting IL-10: Steidler *et al* described the use of transgenic *Lactococcus lactis* capable of secreting bioactive IL-10 in both the DSS and IL-10 knockout models of colitis[84,85]. The authors observed an inhibition of spontaneous colitis development in IL-10 knockout mice that was mediated by relatively low concentrations of the Lactococcus-borne cytokine. These experiments provide the basis for the use of genetically modified organisms designed for delivery of biologically relevant therapeutic molecules.

Synbiotics

A synbiotic is a combination of one or more probiotics and prebiotics[86-88]. Prebiotics may enhance the survival of probiotic strains, as well as stimulating activity of the host' s endogenous bacteria. Bengmark suggests that clinical effects vary from modest effects to significant effects as one goes from single-strain of probiotics < multistrain probiotics < or - < single-strain/single fiber synbiotics < multistrain/multifiber synbiotics[87]. Kanamori *et al* reported that combination therapy with *Bifidobacterium breve*, *Lactobacillus casei,* and galactooligosaccharides dramatically improved the intestinal function in a girl with short bowel syndrome[89]. Roller *et al* demonstrated that combination of oligofructose-enriched inulin combined with *Lactobacillus* and *Bifidobacterium* suppressed colon carcinogenesis by modulating functions of gut-associated lymphoid tissue[90]. They also reported that same synbiotic formula stimulated secretion of secretory-IgA and IL-10 production in the cecum[91]. A symbiotic preparation of *Bifidobacterium* combined with galacto-oligosaccharides protects against Salmonella infection in mice[92]. Thus, synbiotics modulate mucosal immune responses and exert anti-inflammatory effects. We are not aware of any trial design to evaluate potential benefit of synbiotics in IBD patients.

Antibiotics

A few trials of antibacterial agents have been conducted in UC with controversial results. Oral vancomycin and intravenous metronidazole were not beneficial in active UC both as single or adjunctive given therapy[93,94]. Tobramycin, a nonabsorbable, gram-negative specificantibiotic , induced a significant improvement in the short term compared to placebo in UC. However, the improvement was lost at long term follow-up. In addition, the association of tobramycin and metronidazole did not implemented the outcome in patients with severe UC treated by conventional therapy (steroids)[95]. In a a, double-blind placebo-controlled trial run on a small sample, the use of rifaximin (a nonabsorbable, wide-spectrum antibiotic) led to a significant improvement in both clinical and endoscopic activity[96].

A more definite role for antibiotics in UC is in the treatment of pouchitis, where conditions are favourable to bacterial overgrowth[2]. It has been suggested that anaerobes induce pouch inflammation, but some investigators have found a relative increase of aerobic bacteria in pouchitis. In pouchitis, *Bacteroides* species are present in low numbers, whereas *E coli* numbers are increased, but not correlated with the degree of inflammation. Luminal pH is increased, enhancing proteolytic enzyme activity and mucin degradation. Current data suggest that dysbiosis of luminal organisms contributes to pouch inflammation in a susceptible host. Treatment with metronidazole, ciproxin and/or rifaximin leads to a significant decrease of the total number of the following anaerobes and aerobes in fecal samples: *Enterococci, Lactobacilli, Bifidovacteria*, and *Bacteroides*[96].

Antibiotics are largely used in clinical practice for treating active CD and provide more satisfactory results. Controlled trials have supported this treatment[97,98]. In a placebo-controlled trial, both low-dose (10 mg/kg per day)

and high-dose (20 mg/kg per day) metronidazole were more effective than a placebo in colonic CD. In a controlled trial to study the combination of metronidazole and ciprofloxacin versus methylprednisolone for active CD, after 12 wk there was no statistically significant difference in remission rates of the two groups. More recently, ciprofloxaxin was shown to be effective as mesalazine in inducing remission in patients with active CD. Antibiotics do have a role in at least a subset of cases; but we must keep in mind that treatment with antibiotics may have some disadvantages, such as non-specific effect on the enteric flora, the possibility of inducing an antibiotic resistance, and the risk of *Clostridium difficile* superinfection.

In conclusion, the pathogenesis of IBD may be associated with imbalance in the intestinal microflora with a relative predominance of "aggressive" bacteria and paucity of "protective" organisms. Manipulation of the intestinal flora may represent a highly physiologic, nontoxic way to prevent and treat IBD. Although these strategies appear promising and may be actually useful in specific settings, more studies are are needed to establish the relevance of these therapies.

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REVIEW

Living donor liver transplantation to patients with hepatitis C virus cirrhosis

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Abstract

Living donor liver transplantation (LDLT) is an alternative therapeutic option for patients with end-stage hepatitis C virus (HCV) cirrhosis because of the cadaveric organ shortage. HCV infection is now a leading indication for LDLT among adults worldwide, and there is a worse prognosis with HCV recurrence. The antivirus strategy after transplantation, however, is currently under debate. Recent updates on the clinical and therapeutic aspects of living donor liver transplantation for HCV are discussed in the present review.

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Key words: Hepatitis C virus; Living donor liver transplantation; Interferon; Rivabirin

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INTRODUCTION

The use of live donors for liver transplantation was initiated more than a decade ago as a solution to the cadaveric donor shortage for pediatric recipients[1]. After the first successful case in an adult patient in 1994[2], this procedure is now widely applied to adult recipients, especially in countries where the availability of brain-dead donors is severely restricted[3] and also in the United States and European countries, due to a critical shortage of cadaveric organs. Improved surgical techniques and the introduction of new immunosuppressive agents have enhanced the long-term results of living donor liver transplantation (LDLT), leading to an increased demand for liver transplantation that exceeds the number of potential donor organs. In initial experiences with adult LDLT in Japan, the most common indication was cholestatic liver disease, including primary biliary cirrhosis and primary sclerosing cholangitis in Japan. The number of LDLT patients indicated for hepatitis C virus (HCV) has recently increased rapidly.

A recent study[4] of deceased donor liver transplantation (DDLT) reported that HCV infection was associated with a 23% increase in mortality and a 30% increase in the rate of graft failure. The poor results might be due to the recurrence of HCV disease in the graft[5]. HCV-induced graft hepatitis and fibrosis/cirrhosis occur in 75% to 80% and 10 % to 30% of recipients, respectively, at 5 years[6,7]. Once liver cirrhosis is established, the cumulative probability of developing clinical decompensation is close to 50% after 1 year and survival after decompensation is extremely short[8]. Cholestatic hepatitis occurs in approximately 10% of patients infected with HCV and leads to accelerated graft failure and death[9]. One of the hottest debates is the possibility of increased severity of recurrent HCV in LDLT patients. The benefit of LDLT might be offset if the outcome of LDLT for HCV patients is worse than that of DDLT. In this review, we describe current trends and controversies in LDLT for patients with HCV. Our results for LDLT and HCV are also reported.

CURRENT STATUS OF LDLT

According to the Japan Liver Transplantation Society[10], the number of adult patients (≥18 years old) is increasing annually, and has reached 300 in 2003. The most common indication for adults has been hepatocellular carcinoma (n = 311), followed by primary biliary cirrhosis (n = 255), and HCV-related cirrhosis without carcinoma (n = 113). The 1, 3, and 5 year survival rates of all the adult patients were 76%, 72%, and 69%, respectively. Those of HCV-positive patients were 76%, 73%, and 65%, respectively.

In the United States in 2000, there was a high level of enthusiasm for adult LDLT, with 49 centers performing at least one LDLT. Overall, in experienced centers, about a third of adults on the waiting list had a potential living donor and half of them had undergone LDLT; thus, LDLT might be applicable for up to 15% of individuals on the list[11]. The enthusiasm was, however, quickly tempered

Table 1 Comparison between LDLT and DDLT for hepatitis C virus cirrhosis

Study			N		Dif[1]	Protocol biopsy	Findings
Author	Year	Institution	LDLT	DDLT			
Gaglio[23]	2003	Colombia U.	23	45	Yes	No	Cholestatic hepatitis in 17% of LDLT and 0% of DDLT (P = 0.001). No significant difference in incidence of Rec.
Shiffman[28]	2003	Virginia Commonwealth U.	22	53	No	Yes	79% patient survival in LDLT and 91% in DDLT during 3 year (NS). No significant difference in inflammation score in liver specimen after 3 years
Russo[29]	2004	UNOS data	279	3955	No	No	87% 1-year patient survival in both.
Thuluvath[30]	2004	UNOS data	207	408	No	No	No significant difference in patient survival (P = 0.6).
Van Vlierberghe[32]	2004	Ghent U.	17	26	No	No	Rec in 35% of LDLT and 38% of DDLT during 1 year (P = 0.1)
Bozorgzadeh[34]	2004	Rochester U.	35	65	No	No	Rec in 77% of LDLT and 72% of DDLT during 1 year (NS), 89% patient survival in LDLT and 75% in DDLT during 39 mo (NS)

[1] Difference in short-term outcomes or severity of virus recurrence between living and deceased donor liver transplantation. Abbreviations: Rec, Virus recurrence; U, University; NS, not significant; UNOS, United Network for Organ Sharing.

by the death of a donor in 2002 in the United States[12]. Since 2001, the number of patients who have undergone LDLT has declined[13]. Currently less than 5% of all adult liver recipients use living donors. By July 2005, 2734 LDLT cases had been performed. There were 1761 adult patients and HCV was the most common indication. HCV is the most common indication for LDLT[14] and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002.

By the end of 2003, 1743 LDLT cases were recorded in the European Liver Transplantation Registry[15]. According to the Transplant Procurement Management[16], the number of LDLT peaked in 2003 and has gradually decreased over recent years. LDLT accounts for approximately 5% of the total liver transplants performed in Europe. Among the 806 LDLT cases from October 1991 to December 2001[17], the overall 5-year graft survival rate was 75%, better for children than for adults (80% *vs* 66% at 3 years). Cirrhosis secondary to HCV infection is a leading indication for LDLT among adults in Europe[18]. The number of LDLT patients is shown in the Table 1.

INDICATIONS

In areas with low deceased donor organ availability, the indications for LDLT are similar to those for DDLT. In contrast, in Western countries, LDLT is conducted in an attempt to alleviate the shortage of donor organs and to decrease the mortality among the patients awaiting transplants. That is, a balance needs to be achieved between the candidate's liver disease severity and the adequacy of a partial graft for transplantation. The candidate's liver disease should be advanced to the extent that transplantation is justified, but the liver disease cannot be so advanced that a partial graft will not provide adequate hepatic mass.

According to Russo's report[19] a substantial proportion of patients were United Network for Organ Sharing (UNOS) status 3 at the time of LDLT (43%). The policy at their centers prior to the implementation of a model for end stage liver disease (MELD)-based allocation was not to proceed with LDLT in patients meeting UNOS status 2A criteria. Their patient survival rate was 57% with an average stay of 23 d in the intensive care unit. In comparison, 1-year patient survival was 82% in DDLT recipients who were UNOS status 2A at the time of transplant[20].

The waiting list mortality increases in patients with advanced liver disease and patients with a MELD score of 25 have a 20% 3-mo mortality[21]. In general, it is uncommon to proceed with LDLT in patients with MELD scores above 25. Thus, depending on the region of the country and the average MELD score at the time of the transplant within the area served by the organ procurement organization, LDLT might offer patients transplantation before they die waiting for a deceased donor liver. The lower MELD score limit with LDLT is more controversial and varies from center to center. Russo[19] commented that they do not proceed with LDLT in candidates with MELD scores under 11.

LDLT AS A RISK FACTOR FOR RECURRENCE OF HCV

One study from Barcelona[22] reported that LDLT patients (n = 22) had younger donors, less graft steatosis, more frequent biliary complications, and earlier and more severe acute hepatitis compared with DDLT (n = 95) patients. A report from Colombia University[23] indicates that cholestatic hepatitis or severe HCV recurrence occurs more frequently in LDLT. These reports indicate that more intensive antiviral therapy might be necessary for recipients of living donor grafts.

The possible causes of HCV recurrence include HLA matching between donor and recipient. Because cellular immune reactions restricted by both HLA class Ⅰ and Ⅱ antigens are involved in the recognition of HCV peptides[24], HLA matching between donor and recipient could potentially increase damage to the graft from recurrent viral infections by facilitating host recognition of viral antigens[6]. Recently, a beneficial effect of a complete HLA-DQ mismatch was reported in 14 patients after transplantation for HCV cirrhosis[25]. Another possible cause might be related to liver regeneration[26], although recent data[27] did not support this hypothesis. *In vitro*, HCV internal ribosome entry site activity and replication are higher in actively dividing cells, and it is possible that

viral translation is enhanced by factors that stimulate the regeneration of hepatocytes. Moreover, there are experimental data suggesting that liver regeneration induces low density lipoprotein receptor expression, which might facilitate HCV entrance into the hepatocytes.

In contrast, comparable data between LDLT and DDLT for HCV was recently reported[28]. Russo and colleagues[29] compared patient and graft survival in recipients transplanted for chronic HCV who received a living donor organ (n = 279) and deceased donor organ (n = 3955) using the UNOS liver transplant database. One-year patient survival was 87% in both groups and 2-year patient survival was 83% and 81% in the living donor group and deceased donor group (P = 0.68), respectively. Similar results (DDLT, n = 480 *vs* LDLT, n = 207) were obtained from another analysis using the UNOS data base[30]. Analyses from the Mayo Clinic[31] and Gent University[32] also demonstrated no negative impacts of LDLT on the results of liver transplantation for HCV-related cirrhosis.

These data should be interpreted with caution, however, because of the important clinical distinction between LDLT and DDLT recipients. At the time of transplantation, the LDLT group recipients are far less sick than their DDLT group counterparts[33]. The LDLT (n = 35) and DDLT (n = 65) data from a single institution, Rochester University, were examined[34]. Patient survival, graft survival, rate of HCV recurrence, severity of HCV recurrence, graft loss from HCV, and interval for HCV recurrence in DDLT and LDLT were similar. It remains unclear, however, whether LDLT is truly disadvantageous compared to DDLT for HCV-positive patients because the number of cases or follow-up duration is not yet sufficient.

According to the data from Russo[29], from 1999 to 2000, the 1-year patient survival in the LDLT group increased from 69% to 90% (P = 0.04), and 1-year graft survival increased from 63% to 79% (P = 0.16). In contrast, in the DDLT group, 1-year patient and graft survival did not substantially change from 1999 to 2000. As a result, 1-year survival rates became similar between the LDLT and DDLT groups in 2000. The results indicated an experience effect and learning curve on outcomes after LDLT for HCV. Therefore, the initial reports indicating poorer results of LDLT might be due to technical problems from a lack of experience. Recent data indicating similar results between LDLT and DDLT might be due to the increased experience with LDLT. The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide some answers to the questions about recurrent HCV after LDLT and DDLT[35].

MANAGEMENT OF HCV

Therapy for reccurrence in DDLT

If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. One strategy might be aggressive treatment for HCV. Treatment of recurrent HCV disease with interferon and ribavirin after DDLT is used in some centers[36-38]. One standard regimen includes interferon-alpha2b (3 MU × 3 per week) and ribavirin (1000 mg/d) for 6 mo. In a recent trial, polyethylene glycol-conjugated interferon therapy was used[35,38-43], with a sustained viral response rate ranging from 13% to 47%.

Preemptive therapy for HCV after DDLT

Preemptive therapy in the early post-transplantation period with interferon either alone or in combination with ribavirin has been attempted in DDLT, although its effectiveness is controversial. In one study, HCV-positive recipients were randomized within 2 wk of transplantation to receive either interferon alone (3 MU × 3 per wk, n = 30) or placebo (n = 41) for 1 year[39]. Only 17 patients could complete 1 year of interferon therapy. Eight patients (27%) in the interferon group and 22 (54%) of the untreated patients had recurrent hepatitis (P = 0.02). Patient and graft survival at 2 years did not differ between the groups, however, and the rate of viral persistence was not affected by treatment.

In another controlled trial[43], 24 recipients were randomized at 2 weeks post-transplantation to receive interferon (3 MU × 3 per wk) or placebo for 6 mo. There were no differences in graft or patient survival. There were no differences between groups in the incidence of histological recurrence or its severity differed between groups. Recurrent HCV was delayed 408 d in treated patients versus 193 d in the control cohort.

In a case series by Mazzaferro[44], 36 recipients were treated with interferon-alpha 2b (3 MU × 3 per wk) and ribavirin (10 mg/kg per d). They started treatment at a median of 18 d after the operation and treatment continued for 11 mo. After a median follow-up of 52 mo, the 5-year patient survival was 88%. Serum HCV RNA clearance was obtained in 12 patients (33%). They did not require further antiviral treatment because of negative HCV RNA in serum and normal liver histology for a median of an additional 36 mo. The former two randomized trials on preemptive interferon monotherapy demonstrated minimal benefits of the drug. In contrast, Mazzaferro reported more encouraging results, although their protocol brings into question how long therapy is needed once embarking on a preemptive strategy.

Re-transplantation

The approach to retransplantation for recurrent HCV varies widely among the transplant centers of DDLT[11]. The results after retransplantation for HCV (45% at 5 years) are poorer than that for other causes[45] (56%, P < 0.001). The patients with recurrent HCV in the early timing and graft failure within the first year have poor outcomes after retransplantation. These individuals should be considered contraindicated for retransplantation. The experience of retransplantation for HCV in LDLT has not been well accumulated.

OUR EXPERIENCE

We performed preemptive therapy for LDLT patients with HCV infection[38]. From 1996 to 2004, 67 patients underwent LDLT for HCV cirrhosis at the Tokyo University Hospital. The patients were 51 men and 16

women and their ages ranged from 23 to 63 years (median 55). The HCV genotype was 1b in 53 patients (79%). Forty-one patients (61%) had hepatocellular carcinoma. All the patients received the same immunosuppressive regimens with tacrolimus and methylprednisolone.

All the patients preemptively received antiviral therapy consisting of interferon α-2b and ribavirin, which was started approximately 1 mo after the operation. The therapy was continued for 12 mo after the first negative HCV RNA test. The standard regimen included interferon α-2b (3 MU × 3 per wk) and ribavirin (800 mg/d) for 6 mo. The patients were then observed without the therapy for 6 mo. The therapy was continued for at least 12 mo even if the HCV RNA test remained positive.

Therapy was discontinued when there was significant leukopenia (< 1500 /mL), thrombocytopenia (< 50000 / mL) despite application of granulocyte colony stimulating factor (Gran®, Sankyo, Co. Ltd., Tokyo, Japan), hemolytic anemia (hemoglobin < 8 g/L), renal dysfunction (serum creatinine > 20 mg/L), depressive psychological status, or general fatigue. The subjects were removed from the protocol if they did not continue the therapy for 12 mo due to adverse effects or could not start the therapy due to early death.

Blood counts and liver function tests were checked every 2 wk for the first month, and at 4 wk intervals thereafter. Serum samples were collected once a month for quantitative HCV RNA detection. Protocol liver biopsy was not performed. The log-rank test was used to compare the survival rate of the HCV-positive patients with the HCV-negative patients who underwent transplantation during the same period (n = 168).

A total of 28 patients were excluded from the analysis; 12 patients were removed from the protocol because of early death (n = 9) or because of drug cessation (n = 3). Another 16 patients are currently on the protocol and were therefore excluded from the analysis. Of the remaining 39 patients, 16 (16/39; 41%) obtained a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, comparable with that of patients negative for HCV (n = 168, 86%).

CONCLUSIONS

LDLT will remain an indispensable therapeutic tool for HCV related end stage liver disease and an alternative to DDLT. The association between LDLT and early HCV recurrence remains to be determined, although most of the recent papers suggest that live donor graft has no effect on short-term outcome or severity of virus recurrence. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol might improve the outcome of LDLT for HCV.

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REVIEW

Oncological problems in pancreatic cancer surgery

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Abstract

Despite the development of more sophisticated diagnostic techniques, pancreatic carcinoma has not yet been detected in the early stage. Surgical resection provides the only chance for cure or long-term survival. The resection rate has increased due to recent advances in surgical techniques and the application of extensive surgery. However, the postoperative prognosis has been poor due to commonly occurring liver metastasis, local recurrence and peritoneal dissemination. Recent molecular-biological studies have clarified occult metastasis, micrometastasis and systemic disease in pancreatic cancer. Several oncological problems in pancreatic cancer surgery are discussed in the present review.

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Key words: Pancreatic cancer; Extended resection; Molecular diagnosis; Micrometastasis; Adjuvant therapy

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INTRODUCTION

Over the past 30 years, the number of deaths in Japan due to pancreatic carcinoma has steadily increased from 4400 to 19 000[1] (Figure 1). It is the fifth most common cause of death due to malignant neoplasms (Figure 1). Regional pancreatectomy for carcinoma of pancreatic head region, introduced by Fortner[2] in 1973, has impressed many Japanese pancreatic surgeons. Consequently, the resection rate has gradually improved, but the postoperative prognosis is still poor in spite of the development of diagnostic modalities such as CT-scan, EUS, MRI and PET. In 1980, the Japan Pancreas Society (JPS) published the first edition of its "General Rules for Surgical and Pathological Studies on Cancer of the Pancreas". The fifth edition was published in 2002. The second English edition was published in 2003[3]. The JPS also started a registration system for pancreatic carcinoma in 1981. According to the data of JPS, the 5-year survival of invasive ductal carcinoma of the pancreas after pancreatectomy is only 13.4%[4] (Figure 2). JPS and UICC stage of invasive cancer and survival after pancreatectomy are shown in Figure 3[4]. Comparison of survival curves according to the stage reveals that stratification is much better in the JPS classification than in UICC classification.

In 1981, we developed an antithrombogenic bypass catheter for the portal vein to decompress portal congestion or prevent hepatic ischemia caused by simultaneous resection of portal vein and hepatic artery[5]. Since then, we have been aggressively performing extensive surgical resections including portal vein resection by the non touch isolation technique[7,8] using this bypass method. The resection rate has been elevated and operative mortality has remarkably decreased. However, the postoperative prognosis is still poor due to high recurrence rate. The problems of surgical therapy for pancreatic cancer are discussed in this review.

ONCOLOGICAL PROBLEMS

Intrapancreatic carcinoma development

The indications for total pancreatectomy or pancreatoduodenectomy in pancreatic head cancer are one of the key problems in pancreatic cancer surgery. It is very important to know how the carcinoma has developed from the pancreatic head to the body or tail. A high incidence of development or multicentricity of the carcinoma of the pancreatic head to the body or tail has been reported[9,10]. However, recent histopathological and immunocytochemical analysis of total pancreatectomy specimens have clarified that carcinoma development from head to body or tail is continuous[11-13]. Therefore, intraoperative quick histopathological diagnosis combined with immunohistochemical staining using frozen section can diagnose intrapancreatic carcinoma development more precisely[14,15].

Table 1 Comparative studies of extended versus standard operation for pancreatic cancer

Author	Yr	Results
Ishikawa *et al*[24]	1988	Retrospective study [standard (n = 37): 9%, 5-Y-S extended (n = 22): 28%, 5-Y-S
Mukaiya *et al*[25]	1998	Retrospective study 77 institutions, 501 patients: NS
Henne-Bruns *et al*[26]	2000	Retrospective study [standard (n = 26) extended (n = 46)] NS
Pedrazzoli *et al*[27]	1998	RCT [standard (n = 40) extended (n = 41) overall survival: NS survival of node positive patients: extended > standard
Yeo *et al*[28]	2002	RCT [standard (n = 146) extended (n = 148) mortality: NS, morbidity: extended > standard, survival: NS

RCT: Randomized controlled test; NS: Not significant.

Lymph node metastasis

Lymph node dissection is one of the important components in pancreatic cancer surgery. The high incidence of 56%[16], 70.5%[17], 73%[18], 76%[19], 77%[20], and 86.4%[21] in resected specimen of pancreatic cancer is the reason for wide dissection of lymph nodes in pancreatic cancer surgery. There are few reports about precise para-aortic lymph node metastasis. The incidence of para-aortic lymph node metastasis for pancreatic head carcinoma is reported to be 16% (7/44)[17] and 26% (23/90), respectively[20]. The incidence of pancreatic body and tail carcinoma is 13% (4/30)[22] and 17% (4/27)[21], respectively. The lymphatic flow from the pancreatic head tumor to the para-aortic lymph node via the posterior surface of the pancreatic head and around the superior mesenteric artery has been suspected[17,18,23].

The efficacy of extended lymph node dissection in pancreatic cancer surgery has been suggested in a retrospective study[24]. However, the efficacy of extended lymph node dissection has not been clarified in retrospective studies[25,26] or in recent prospective randomized controlled tests for pancreatic cancer surgery (Table 1)[27,28].

The incidence of perigastric lymph node metastasis in pancreatic cancer is relatively low[20]. Therefore, pylorus preserving pancreatoduodenectomy (PPPD) is indicated for pancreatic head carcinoma, although its advantage over the classic Whipple operation has not been clarified[29,30].

Vascular invasion

Portal vein resection is another problem in pancreatic cancer surgery. To prevent portal congestion in portal vein resection and hepatic ischemia in simultaneous resection of portal vein and hepatic artery, we developed a catheter-bypass procedure[5,6] in our department in 1981 using antithrombogenic catheter, and isolated pancreatectomy combined with portal vein resection has thus been established[8]. During the past 30 years, the operative mortality rate of pancreatoduodenectomy combined with portal vein resection has decreased, and portal vein resection in pancreatic cancer surgery has become a safe operative procedure. The reported mortality rate is 7.4% (2/27)[31], 10% (6/63)[32], 5% (3/58)[33], 0% (0/31[34], 0/14[35], 0/34[36], 0/24[37]), and 3.2% (1/31)[38]. From 1981 to 2003, 250 of 391 (63.9%) patients with pancreatic carcinoma underwent tumor resection in our department. Portal vein resection was performed in 171 of these 250 (68.4%) resected cases, and the mortality rate was 4.4% (11/250)[39]. The indication and contraindication for portal vein resection have not yet been clarified in pancreatic cancer surgery. There are many reports about the benefit[33,34,40] or no benefit[41] of portal vein resection for curative resection or survival. The most important indication for portal vein resection in pancreatic cancer is the ability to obtain cancer-free surgical margins[39].

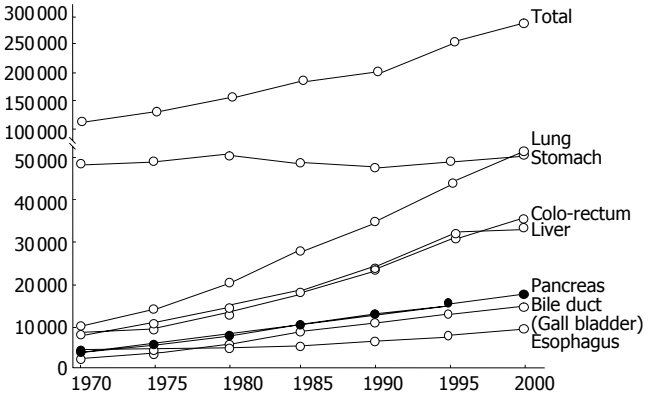

Figure 1 Trends in death due to malignant neoplasms in Japan.

In severe portal invasion cases, it is difficult to obtain cancer-free surgical margins, so the prognosis is poor[39,42-44]. A recent diagnostic modality using intraportal endovascular ultrasonography provides precise information about the relationship between the pancreatic cancer and the portal vein wall, and planning of the operative procedure[45-47].

Extrapancreatic nerve plexus invasion

Pancreatic carcinoma often invades the extrapancreatic nerve plexus[48-51]. There is continuity of the intrapancreatic neural invasion into the extrapancreatic nerve plexus[48]. The grade of intrapancreatic neural invasion correlates with the extrapancreatic nerve plexus invasion[50,51] and the manner of neural invasion has no relationship with the behavior of lymph node metastasis[50].

In pancreatic head carcinoma, complete dissection of extrapancreatic nerve plexus , especially the second portion of pancreatic head nerve plexus and nerve plexus around the superior mesenteric artery, is sometimes necessary to obtain a carcinoma-free surgical margin. However, complete resection of the nerve plexus around

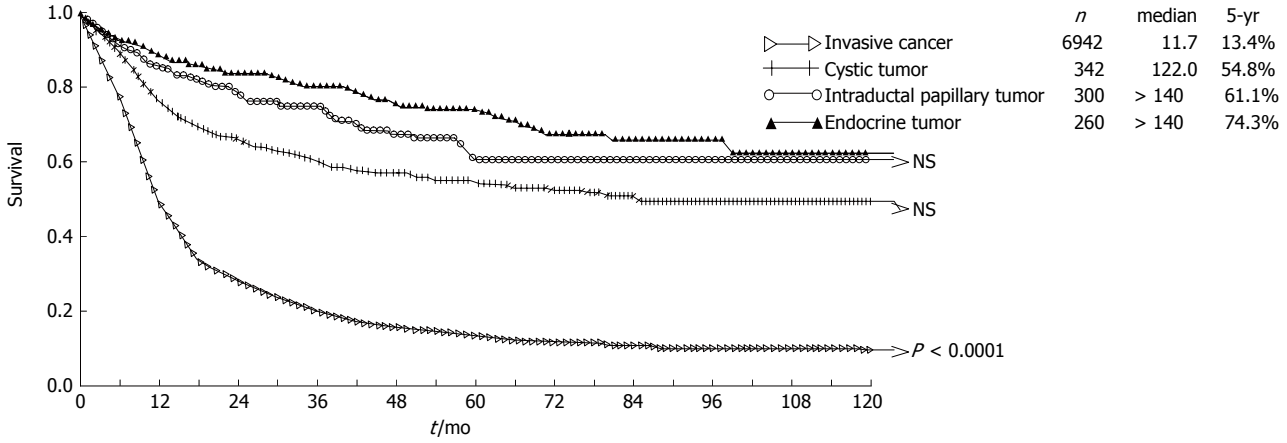

Figure 2 Histology and survival after pancreatectomy. Survival of patients who underwent pancreatectomy is shown. NS, not significant.

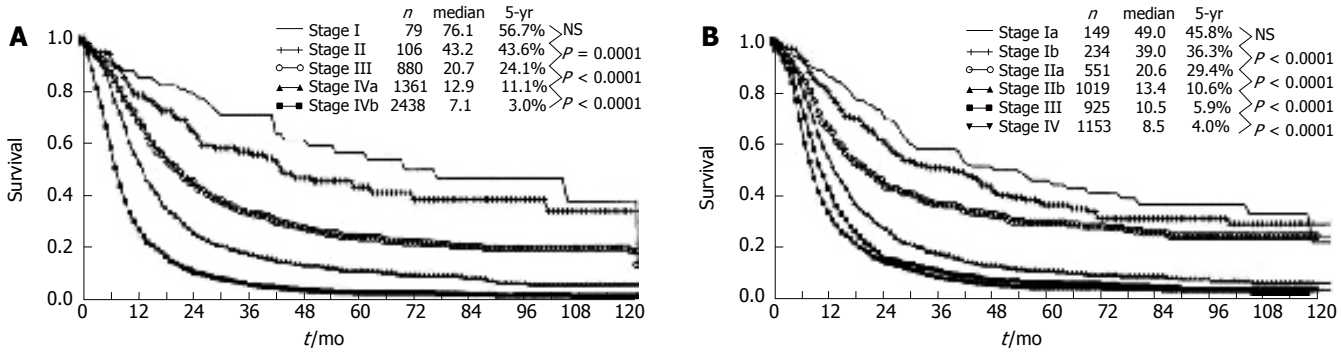

Figure 3 Survival after pancreatectomy according to JPS stage (**A**) and UICC stage (**B**). NS, not significant.

the superior mesenteric artery causes severe diarrhea after surgery, and the prognosis of positive carcinoma invasion to the extrapancreatic nerve plexus cases is very poor[39,50,51]. The greatest cause of carcinoma-positive surgical margin is extrapancreatic nerve plexus carcinoma invasion[39,48,50]. Recently, carcinoma invasion to the second portion of the pancreatic head nerve plexus can be diagnosed using intraportal endovascular ultrasonography[45-47,52]. In our department, if patients have no carcinoma invasion to the second portion of the pancreatic head nerve plexus, the left semi-circular nerve plexus around the superior mesenteric artery is preserved to prevent postoperative diarrhea.

Postoperative recurrence

Even in extended surgery, a high incidence of postoperative liver metastasis, local recurrence, and peritoneal metastasis has been observed with a poor postoperative prognosis (Table 2)[53-57]. The precise diagnosis of recurrence type is difficult even if modern diagnostic modalities are used. However, the local recurrence was 100% and the liver metastasis was 80% in 25 autopsy cases[55]. The first cause of poor postoperative prognosis in pancreatic cancer is liver metastasis. Although occult liver metastasis may be suspected on the basis of extensive clinical data, no criteria have been definitely determined. Surgical therapy combined with effective adjuvant therapy is necessary in view of these types of recurrence.

Adjuvant therapy

Surgical therapy currently offers the only potential cure for pancreatic cancer. However the recurrence rate is very high and the long-term survival is poor.

The potential benefit of adjuvant therapy after resection of pancreatic cancer was first recognized by the randomized trial conducted by the Gastrointestinal Tumor Study Group (GITSG) using chemoradiotherapy almost 20 years ago[58,59]. Since then, few randomized trials have shown a benefit of adjuvant treatment (Table 3)[60-65]. The study of the European Study Group for Pancreatic Cancer (ESPAC-1) concluded that postoperative chemotherapy with fluorouracil plus leucovorin confers a benefit in terms of survival, whereas postoperative chemoradiotherapy has a deleterious effect on survival[64]. The current study by Neuhaus *et al*[65] indicates that the treatment with gemcitabine in patients with resected pancreatic cancer can result in improved disease-free survival as compared to observation.

A new and more effective adjuvant therapy must be established by prospective randomized trials using newly developed drugs[66,67] or therapeutic modalities[68]. Nevertheless, the individualized adjuvant therapy is very important in pancreatic cancer treatment[69,70].

Occult metastasis and micrometastasis

Recent progress in immunohistochemistry and molecular biological studies has made it possible to clarify the occult metastasis and micrometastasis in pancreatic cancer. The

Table 2 Incidence of postoperative recurrence in pancreatic cancer

Author	Yr	Cases (*n*)	Liver (%)	Local (%)	Peritoneal (%)	Bone (%)	Lung (%)	Other (%)
Westerdahl *et al*[53]	1993	74	92	86.5				
Kayahara *et al*[54]	1993	30	60	83.3	40			
Takahashi *et al*[55]	1995	25	80	100	56	24	56	
Sperti *et al*[56]	1997	78	62	72	6			
Nakao *et al*[57]	1997	76	57	34	41	3	1	1

Table 3 Randomised controlled trials of adjuvant treatment for pancreatic ductal adenocarcima

Trial	Comparison	Adjuvant treatment	Number of patients	Conclusions
GITSG, 1985[58], 1987[59]	CRT *vs* OBS	2 × (20 Gy in 10 fractions + 500 mgm^{-2} 5FU d 1-3) + weekly 5FU to recurrence	49 pancreatic patients randomised	Significant increase in median survival (20 *vs* 11 mo, $P = 0.035$) in 43 eligible patients
Norway, 1993[60]	CT *vs* OBS	AMF (40 mgm^{-2} doxorubicin, 6 mgm^{-2} mytomycin C, 500 mgm^{-2} 5FU) once every 3 wk for six courses	61 patients (47 pancreatic, 14 ampullary) randomised 46 additional nonrandomised patients	Significant increase in median survival (23 *vs* 11 mo, $P = 0.02$) in 60 pancreatic and ampullary patients combined
EORTC, 1999[61]	CRT *vs* OBS	2 × (20 Gy in 10 fractions + 25 $mgkg^{-1}$ 5FU/FA d 1-5)	218 patients (120 pancreatic, 93 ampullary) randomised	NS increase in median survival (25 *vs* 19 mo, $P = 0.21$) in 207 eligible patients NS increase in median survival in 114 eligible pancreatic patients (17 *vs* 13 mo, $P = 0.099$)
Japan, 2002[62]	CT *vs* OBS	6 mgm^{-2} mytomycin C d 1 + 310 mgm^{-2} 5FU d 1-5 and d 15-20 followed by 100 mgm^{-2} oral 5FU daily until recurrence	508 patients (173 pancreatic, 335 bile duct/gallbladder/ ampullary) randomised	Significant survival benefit in gallbladder No difference in 158 eligible pancreatic patients No difference in 48 eligible ampullary patients
ESPAC1, 2001[63], 2004[64]	CRT *vs* no CRT CT *vs* no CT	2 × (20 Gy in 10 fractions + 500 mgm^{-2} 5FU/FA d 1-3) (20 mgm^{-2} FA + 425 mgm^{-2} 5FU d 1-5) × six cycles	289 pancreatic patients randomised	NS decrease in survival for CRT ($P = 0.05$) in 289 patients Significant increase in survival for CT ($P = 0.009$) in 289 eligible patients
CONKO-001, 2005[65]	CT *vs* OBS	1 gm^{-2} GEM, d 1, 8, 15, every 4 wk for 6 mo	368 pancreatic patients randomised	Significant increase in median DFS (14.2 *vs* 7.5 mo, $P < 0.05$) in 356 eligible patients

CRT: Chemoradiotherapy; CT: Chemotherapy; OBS: Observation; NS: Not significant; DFS: Disease-free survival.

Table 4 Incidence of pancreatic cancer cells in peripheral blood, bone marrow, and liver tissue

Author	Yr	Incidence
Tada *et al*[71]	1993	Peripheral blood, K-*ras* 2/6 (33%)
Juhl *et al*[72]	1994	Bone marrow, immunostaining: 15/26 (58%)
Inoue *et al*[73]	1995	Liver tissue, K-*ras*: 13/17 (76%)
Nomoto *et al*[74]	1996	Peripheral blood, K–*ras*: postoperative period 10/10 (100%)
Funaki *et al*[75]	1996	Peripheral blood, CEAmRNA: 3/9 (33%)
Aihara *et al*[76]	1997	Peripheral blood, Keratin 19m RNA: 2/38 (5%)
Miyazono *et al*[77]	1999	Peripheral blood, CEAmRNA: 13-21 (61.9%)
Uemura *et al*[78]	2004	Peripheral blood, K-*ras*: 9/26 (35%)

Table 5 Reports of occult lymph node metastasis

Author	Yr	Results
Tian *et al*[79]	1992	HE: 8/56 (14%) Cytokeratin: 17/56 = (30%)
Ando *et al*[80]	1997	K-*ras*: paraaortic lymph nodes: 42/101 (42%)
Demeure *et al*[81]	1998	K-*ras*: Stage I (T1-2, N0, M0) 16/22 (73%)
Yamada *et al*[82]	2000	K-*ras* (-) has a better prognosis than K-*ras* (+)
Bogoevski *et al*[83]	2004	Ber–EP4: immunostaining 56/148 (37.8%)

Table 6 Incidence of occult peritoneal dissemination

Author	Yr	Results
Lei *et al*[84]	1994	Peritoneal washings, conventional cytology, 3/36 (8%), 1/11 (9%) with ascites
Juhl *et al*[72]	1994	Immunostaining (CEA, CA19-9,…, cytokeratin bone marrow 58%, peritoneal washings 58%
Vogel *et al*[85]	1999	Peritoneal washings 39%, bone marrow 38%, one of them positive: died within 19 mo, both negative: 5 y.s. 30% ($P < 0.0001$)
Castillo *et al*[86]	1995	Laparoscopy 16/94 (17%)
Leach *et al*[87]	1996	4/60 (7%)
Nomoto *et al*[88]	1997	Conventional: 0/18 (0%), immunostaining (CEA, CA19-9): 2/18 (11%)
Nakao *et al*[89]	1999	Conventional: 5/66 (8%), immunostaining 14/66 (22%) prognosis between cytology positive and negative: NS

high incidence of K-*ras* point mutation of codon 12 in pancreatic cancer has been observed. Occult pancreatic cancer cells have been detected in peripheral blood, bone marrow and liver by studies of K-*ras*, CEA mRNA, keratin 19 mRNA, along with immunocytochemical staining (Table 4)[71-78].

Occult lymph node metastasis in pancreatic cancer has been also detected by the studies of K-*ras* and immunostaining of cytokeratin or Ber-FP4 (Table 5)[79-83].

The incidence of cancer cells from abdominal washing cytology is shown in Table 6[84-89]. The incidence using conventional staining is 0%-17% (Table 6)[84,86-89]. However

a high incidence of 58%[72], 39%[85], and 22%[89] by immunocytochemical staining using monoclonal antibodies against tumor-associated antigens and cytokeratins has been reported. The difference in prognosis between positive and negative occult metastases remains controversial.

CONCLUSION

Surgical techniques for pancreatic cancer have been developed, and the resection rate has increased in Japan over the past 30 years. However, the prognosis of stage IV patients with pancreatic cancer is still poor even after aggressive surgery because of its high recurrence rate. Occult metastasis and micrometastasis have been more precisely diagnosed by immunocytochemical and molecular biological studies. On the basis of such data, adjuvant multimodal therapies targeting occult metastasis and micrometastasis with radical surgery are recommended. The effectiveness of these adjuvant multimodal therapies must be clarified and more effective adjuvant therapies must be developed.

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Diagnostic role of serum interleukin-18 in gastric cancer patients

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Abstract

AIM: To determine the current status in various aspects of gastric cancer patients and to find out the clinical correlation with prognostic role of serum interleukins in Thai patients.

METHODS: Sixty-eight patients were enrolled in this study at King Chulalongkorn Memorial Hospital during April 2003 to May 2005. Gastric cancer was histologically proven in 51 patients and gastric ulcer in 17 patients. Serum IL-6, IL-10, IL-12, and IL-18 levels were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS: There were 26 males (55.32%) and 21 females (44.68%) with their age ranging from 33 to 85 years (mean age 64.49 ± 13.83 years). The common presentations were weight loss (41.2%), dyspepsia (39.2%), and upper gastrointestinal bleeding (15.7%). A total of 35.3% gastric cancer patients and 6.3% of gastric ulcer patients were smokers (P = 0.029). Moreover, 32.4% of gastric cancer patients and 6.3% of gastric ulcer patients were alcoholic drinkers (P = 0.044). Lesion location was pyloric-antrum in 39.4%, gastric body in 39.4%, upper stomach in 12.2% and entire stomach in 6.1% of the patients. *H pylori* infection was detected in 44.4%. The poorly-differentiated adenocarcinoma was the most common pathologic finding (60.7%). Surgical treatment was performed in 44.1% patients (total gastrectomy in 5.9%, subtotal gastrectomy in 32.4% and palliative bypass surgery in 5.9%). Systemic chemotherapy was given as an adjuvant therapy in 8.8% patients. Carcinomatosis peritoneii were found in 18.8% patients. The mean survival time was 13.03 ± 9.75 mo. The IL-18 level in gastric cancer patient group (58.54 ± 43.96 pg/mL) was significantly higher than that in gastric ulcer patient group (30.84 ± 11.18 pg/mL) (P = 0.0001) (95% CI was 42.20, 13.19). The cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL, the positive predictive value was 92.31%. The IL-6 level in gastric cancer patients with distant metastasis (20.21 ± 9.37 pg/mL) was significantly higher than that in those with no metastasis (10.13 ± 7.83 pg/mL) (P = 0.037) (95% CI was 19.51, 0.65). The role of IL-10 and IL-12 levels in gastric cancer patients was to provide data with no significant difference.

CONCLUSION: These findings demonstrate that serum IL-6 and IL-18, but not IL-10 and IL-12 levels may be the useful biological markers of clinical correlation and prognostic factor in patients with gastric cancer. Moreover, IL-18 could serve as a diagnostic marker for gastric cancer with a high positive predictive value.

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Key words: Interleukin; Gastric cancer; Diagnostic marker

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INTRODUCTION

The most recent estimates of the world-wide incidence of cancer indicate that gastric cancer is the second most frequent cancer in the world after lung cancer, with over 900 000 new cases diagnosed every year[1].

For the risk factors, many gastric ulcer patient studies conducted all over the world have shown that the elevated risk is associated with consumption of canned fruit, pickled and smoked foods[2,3]. Many studies have found a positive association between tobacco use and relative risk among heavy drinkers as compared to non-drinkers and gastric carcinoma[4,5]. *H pylori*, a Gram-negative spiral-shaped bacterium, has been established as a major etiologic

agent of chronic gastritis and peptic ulcer diseases including duodenal ulcer (DU) and gastric ulcer (GU). The role of *H pylori* infection in gastric adenocarcinoma and MALT is also increasingly recognized[6,7].

The metastatic process of gastric cancer consists of tumor cell detachment, local invasion, motility, angiogenesis, vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in different organs. In each step, causative molecules have been identified including cell-adhesion molecules, various growth factors, matrix degradation enzymes, and motility factors. Most of these molecules can be regarded as prognostic factors[8].

Human IL-6 consists of 212 amino acids, including a hydrophobic signal sequence of 28 amino acids. Many different types of lymphoid and non-lymphoid cells produce IL-6, which is involved in the following multiple biological activities. IL-6 has a strong activity in stimulating the growth of human gastric cancer cell lines. These findings suggest that IL-6 may play a potential role in the pathogenesis of gastric cancer[9].

IL-10 is an 18 Ku peptide and comprises 178 amino acids. This cytokine was first identified as an anti-inflammatory cytokine. The function of IL-10 is to act on the macrophages, inhibiting synthesis and suppressing gene of other cytokines[10].

IL-12 was originally identified as a natural killer (NK) cell stimulatory factor, a disulfide-linked heterodimeric cytokine composed of 35 and 40 Ku subunits. IL-12, secreted principally by antigen presenting cells (APC), such as macrophages, B cells, and dendritic cells, activates NK cells and T cells to produce interferon-γ (IFN-γ), and augments their cytotoxic activity and proliferation. IL-12 has been recently found to induce anti-tumor effects against a variety of tumors *in vivo*[11].

IL-18, formerly called interferon-γ-inducing factor, is a recently discovered cytokine that plays an important role in the TH1 response. IL-18 is produced by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, and osteoblasts. Numerous investigations have noted the importance of IL-18 as a TH1 cytokine, especially in cooperation with IL-12, in anti-tumor immunity[12,13].

Therefore, the purpose of this study was to investigate the possible involvement of serum IL-6, IL-10, IL-12, and IL-18 levels in determining clinicopathologic features and diagnostic yield as well as in predicting the spread of tumors, and most notably including the outcome measured as survival duration for patients with gastric carcinoma.

MATERIALS AND METHODS

Patients and blood samples

There were 68 patients enrolled in this study at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during April 2003 to May 2005. Gastric cancer was histologically proven in 51 patients and 17 gastric ulcer patients served as control. The Ethical Committee of the Faculty of Medicine, Chulalongkorn University, approved the study. Written informed consent was obtained from all patients. Immediately after blood sampling, serum was obtained by centrifugation at 2000 r/min for 15 min at 4℃ and stored at 80℃ until later analysis. Serum IL-6, IL-10, IL-12 and IL-18 levels were determined using ELISA kits (Quantikine R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Briefly, serum samples were reacted with a monoclonal antibody that recognized an epitope of rat interleukins. After 2 h incubation and washing, streptavidin peroxidase-conjugated monoclonal antibody directed to a second epitope was added. This antibody bound to the interleukins captured by the first monoclonal antibody. The color reaction was terminated by a stop solution containing sulfuric acid, and absorbance was measured at 450 nm. Results were calculated from a standard curve generated by a four-parameter logistic curve-fit and expressed in pg/mL.

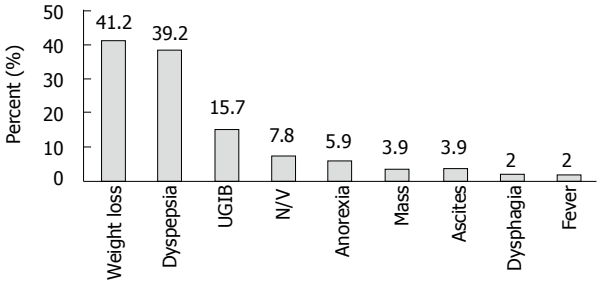

Figure 1 Symptoms of gastric cancer patients.

The clinical data collected included age, sex, symptoms, presentation, history of cigarette smoking and alcohol drinking, lesion site, histological type, metastasis, treatment, *H pylori* status, blood chemistry, result of treatment, and survival time. The histological diagnosis was based on the morphological examination by hematoxylin and eosin-staining.

Statistical analysis

Data were expressed as mean ± SD. Comparisons between groups were analyzed by the chi-square test for categorical variables and Student's *t* test with 95% confidence interval when appropriate for quantitative variables. Survival curves were constructed using the Kaplan-Meier method. The Pearson correlations were used. *P* values below 0.05 for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for Windows, version 11.5.

RESULTS

Of the 51 patients with gastric cancer, 26 were males (55.32%) and 21 were females (44.68%) with their age ranging from 33 to 85 years (mean age 64.49 ± 13.83 years). The common presentations included weight loss (41.2%), dyspepsia (39.2%), and upper gastrointestinal bleeding (15.7%) (Figure 1).

A total of 35.3% of gastric cancer patients and 6.3% of controls were smokers ($P = 0.029$). Moreover, 32.4% of gastric cancer patients and 6.3% of controls were alcoholic drinkers ($P = 0.044$).

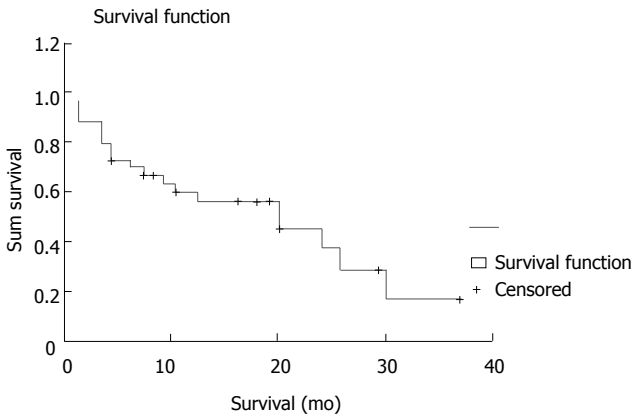

Figure 2 Mean survival time of gastric cancer patients.

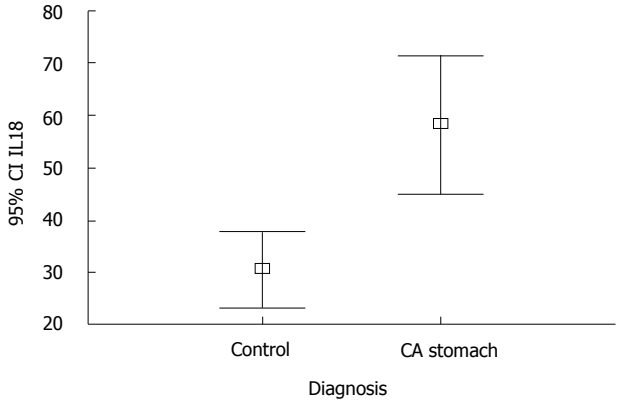

Figure 3 IL-18 levels in gastric cancer patients compared with gastric ulcer patients.

Lesion location was found in pyloric-antrum of 39.4% patients, gastric body of 39.4% patients, upper stomach of 12.2% patients and entire stomach of 6.1% patients. *H pylori* infection was detected in 44.4% patients. The poorly differentiated adenocarcinoma was the most common pathologic finding (60.7%). Surgical treatment was performed in 44.1% patients (total gastrectomy in 5.9%, subtotal gastrectomy in 32.4% and palliative bypass surgery in 5.9%). Systemic chemotherapy was given as an adjuvant therapy in 8.8% patients. Carcinomatosis peritoneii were found in 18.8% patients. The mean survival time was 13.03 ± 9.75 mo (Figure 2).

The IL-18 level in gastric cancer patient group (58.54 ± 43.96 pg/mL) was significantly higher than that in control group (30.84 ± 11.18 pg/mL) (P = 0.0001) (95% CI was 42.20, 13.19) (Figure 3). From the ROC curve, the cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL. The sensitivity was 52.17%, the specificity was 83.33%, and the positive predictive value was 92.31%.

The IL-6 level in gastric cancer patients with distant metastasis (20.21 ± 9.37 pg/mL) was significantly higher than that in those with no metastasis (10.13 ± 7.83 pg/mL) (P = 0.037) (95% CI was 19.51, 0.65) (Figure 4).

A significant clinical correlation was found between hematocrit level, serum albumin, survival time, and serum IL-6, IL-10 and IL-18 levels in gastric cancer patients (Table 1). There was no significant correlation between *H pylori* status and interleukin levels.

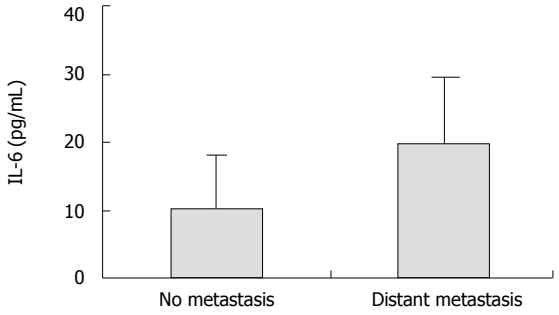

Figure 4 IL-6 levels in gastric cancer with distant metastasis compared to those with no metastasis.

Table1 Correlation between clinical data and serum interleukins in gastric cancer

Correlations	Age	Hct	Alb	Survival (mo)	IL-6	IL-10	IL-12	IL-18
Age	1.000	0.124	-0.104	0.266	0.340	-0.135	0.122	0.109
Hct	0.124	1.000	0.593[2]	0.337[1]	-0.466[2]	-0.096	0.163	-0.481[2]
Alb	-0.104	0.593[2]	1.000	0.473[1]	-0.794[2]	-0.500[2]	-1.000[2]	-0.655[2]
Survival (mo)	0.266	0.337[1]	0.473[1]	1.000	-0.161	-0.011	0.125	-0.133
IL-6	0.034	-0.466[2]	-0.794[2]	-0.161	1.000	0.182	-0.243	0.262[1]
IL-10	-0.135	-0.096	-0.500[2]	-0.011	0.182	1.000	-0.598	0.200
IL-12	0.122	0.163	-1.000[2]	0.125	-0.243	-0.598	1.000	-0.814
IL-18	0.109	-0.481[2]	-0.655[2]	-0.133	0.262[1]	0.200	-0.814	1.000

[1,2] Pearson correlation are both significant at 0.01 level (2-tailed).

DISCUSSION

Gastric carcinoma is one of the most common malignant diseases worldwide. Although its incidence has declined dramatically in the United States of America and Western Europe over the past 60 years, the incidence still remains very high in developing countries[14]. The factors leading to this variability among countries are still not clear. Some correlation exists between the occurrence of gastric carcinoma and the prevalence of *H pylori* infection in different geographical areas[15]. Current knowledge of the detailed mechanisms underlying the interplay between biological modulators and lesions induced by *H pylori* is still incomplete. It is believed that chronic infection with *H pylori* leads to alterations of the cell cycle, including increased epithelial cell replication, increased rate of cell death (apoptosis) and production of oxidants[16,17]. In this study, the prevalence of *H pylori* was 44.4%. There was no significant correlation between *H pylori* status and interleukin levels.

In agreement with other studies, our study examined the effect of cigarette smoking and alcohol drinking as a risk factor for gastric cancer. From the mechanism viewpoint, the direct carcinogenic effect of cigarette smoking may derive from precursor gastric lesions. Indirect effects of inhaled cigarette smoking may involve

the nitrosamines in stomach. Alcohol could act as a contributory factor by causing chronic irritation of the gastric mucosa[4,5].

Weight loss and dyspepsia were the most frequent initial symptoms in our patients, being consistent with those in Western studies[18,19]. The current data indicate that the proportion of early, curable gastric cancer is small among Thai patients. Most of the cases in our study were in advanced stage (stages Ⅲ and Ⅳ) with a very poor 5-year survival.

IL-18 is synthesized as an inactive precursor (pro-IL-18, 24 Ku), which is cleaved by IL-1β-converting enzyme (ICE or caspase-1) into an active 18 kDa mature form[20,21]. IL-18 has multiple biological activities *via* its capacity of stimulating innate immunity and both Th1 and Th2-mediated responses. It also exerts anti-tumor effects that are mediated by enhancement of NK cell activity, reduction of tumorigenesis, induction of apoptosis and inhibition of angiogenesis in tumor cells[22,23]. In addition, recent data suggest that an inappropriate production of IL-18 contributes to the pathogenesis of cancers and may influence the clinical outcome of patients[24]. This is the first study demonstrating that the levels of serum IL-18 are significantly elevated in gastric cancer patients compared with gastric ulcer patients. Although a highserum IL-18 level was not used as a significant prognostic factor in terms of overall survival, it was used as a diagnostic factor. The cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL. The sensitivity was 52.17%, the specificity was 83.33%, the positive predictive value was 92.31%. The pathways for IL-18 production and its mechanisms of action in patients with gastric cancer remain to be determined.

IL-6 is involved in many biological activities, including T-cell and B-cell growth and differentiation, IL-2 production and IL-2 receptor expression, hematopoietic stem cell growth, megakaryocyte maturation, acute phase protein synthesis, macrophage differentiation, mesangial cell, keratinocyte, and osteoclast cell growth, hybridoma, plasmacytoma, and myeloma growth, and stimulation of cancer-cell growth[25]. Our data show that the IL-6 level in gastric cancer patients with distant metastasis is significantly higher than that in those with no metastasis. As a proof of the mechanism of IL-6 action, Tamm *et al*[26] showed that IL-6 makes cancer cells increase their motogenic activity by autocrine pathway. IL-6 secreted from the cancer cells combines with IL-6, which is expressed on the surface of cancer cells, IL-6 and IL-6r act on the cancer cells directly. IL-6 may act through HGF on cancer cells by promoting and accelerating invasion as well as lymph node and/or hepatic metastasis[27].

IL-12 is an immunoregulatory cytokine that triggers the development of a specific T cell-mediated immune response. IL-12 enhances the proliferation, cytokine production, and cytotoxic activity of T lymphocytes and NK cells, with consequent anti-tumor activity[11]. Lissoni *et al*[28] reported that serum IL-12 level is significantly higher in patients with metastatic renal cell carcinoma and breast cancer than in those with local solid neoplasms. Uno *et al*[29] reported that phytohemagglutinin-induced production of IL-12 is lower in patients with gastric cancer than in healthy controls, by examining peripheral blood mononuclear cells *in vitro*. In this study, we could not demonstrate any significant uses of IL-12 in gastric cancer patients as compared with gastric ulcer patients. Shibata *et al*[30] reported that the production of IL-12 decreases significantly with advancing stages, and is the lowest in patients with distant metastases and cachexia.

For IL-10 level, the data indicate that intracellular IL-10 status on monocytes in patients with advanced gastric cancer is significantly increased compared with those with early disease or healthy individuals[31]. In this study, we also found a correlation between IL6, IL-10, and IL-18 levels and serum albumin. In our previous report, serum albumin level is another prognostic factor in gastric cancer at initial diagnosis, and the median survival time is reduced with a decrease in serum albumin level[32].

In conclusion, our data demonstrate that serum IL-6 and IL-18, but not IL-10 and IL-12 levels, may be the useful biological markers for clinical correlation and prognostic factor in patients with gastric cancer. Moreover, IL-18 could serve as a diagnostic marker for gastric cancer with a high positive predictive value. Thus, the detailed mechanisms of IL-6 and IL-18 involving tumor progression should be further investigated.

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LIVER CANCER

Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells

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Abstract

AIM: To investigate the effect of hot water-extracted *Lycium barbarum* (LBE) and *Rehmannia glutinosa* (RGE) on cell proliferation and apoptosis in rat and/or human hepatocellular carcinoma (HCC) cells.

METHODS: Rat (H-4-II-E) and human HCC (HA22T/VGH) cell lines were incubated with various concentrations (0-10 g/L) of hot water-extracted LBE and RGE. After 6-24 h incubation, cell proliferation (n = 6) was measured by a colorimetric method. The apoptotic cells (n = 6) were detected by flow cytometry. The expression of p53 protein (n = 3) was determined by SDS-PAGE and Western blotting.

RESULTS: Crude LBE (2-5 g/L) and RGE (2-10 g/L) dose-dependently inhibited proliferation of H-4-II-E cells by 11% (P < 0.05) to 85% (P < 0.01) after 6-24 h treatment. Crude LBE at a dose of 5 g/L suppressed cell proliferation of H-4-II-E cells more effectively than crude RGE after 6-24 h incubation (P < 0.01). Crude LBE (2-10 g/L) and RGE (2-5 g/L) also dose-dependently inhibited proliferation of HA22T/VGH cells by 14%-43% (P < 0.01) after 24 h. Crude LBE at a dose of 10 g/L inhibited the proliferation of HA22T/VGH cells more effectively than crude RGE (56.8% ± 1.6% *vs* 70.3% ± 3.1% of control, P = 0.0003 < 0.01). The apoptotic cells significantly increased in H-4-II-E cells after 24 h treatment with higher doses of crude LBE (2-5 g/L) and RGE (5-10 g/L) (P < 0.01). The expression of p53 protein in H-4-II-E cells was 119% and 143% of the control group compared with the LBE-treated (2, 5 g/L) groups, and 110% and 132% of the control group compared with the RGE -treated (5, 10 g/L) groups after 24 h.

CONCLUSION: Hot water-extracted crude LBE (2-5 g/L) and RGE (5-10 g/L) inhibit proliferation and stimulate p53-mediated apoptosis in HCC cells.

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Key words: *Lycium barbarum* extract; *Rehmannia glutinosa* extract; Proliferation; Apoptosis; Hepatocellular carcinoma

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INTRODUCTION

According to the official report by the Department of Health, Taiwan, malignant tumor is the first leading cause of death in 2004. Among cancers, hepatocellular carcinoma (HCC) is the second leading cause of death. The mortality rate for HCC is 31.17 per 100 000, accounting for 17.9% of cancer deaths in 2004. The rising incidence of HCC in at-high-risk patients with chronic hepatitis B or C is an important issue in Taiwan. Although early diagnosis and treatment improve survival, HCC is rarely cured and recurs frequently after regional therapy or transplantation[1]. Recently, preventing HCC formation and HCC therapy are major research focuses.

Both *Lycium barbarum* (LBE) *L Rehmannia glutinosa* (RGE) have been commonly used as traditional Chinese medicine and herbal foods for health promotion in China. The active components of the fruit of LBE *L* the dried root of RGE primarily contain water-soluble polysaccharides. LBE *L* RGE can be extracted with hot water followed by precipitation with ethanol to obtain high quantity of polysaccharides[2-4]. Polysaccharide-containing active components purified from these herbs have been recently studied for their physiological and pharmaceutical activities. LBE polysaccharides as glycopeptides isolated

from the fruit of LBE[5,6] are water soluble and potent in immunomodulation, anti-lipid peroxidation[6-9], and antitumor[3]. RGE polysaccharides isolated from the dried root of RGE have also shown the properties of immunomodulation[10-14] and antitumor[15]. To few studies have investigated the effect of LBE and RGE extracts on HCC. The purpose of this study was to investigate the effect of crude hot water-extracted LBE and RGE on cell proliferation and apoptosis in HCC cells.

MATERIALS AND METHODS

Preparation of crude herbal extracts

LBE *L Radix* RGE (processed and dried RGE) were purchased from Chien Yuan Hang (Taipei, Taiwan). To maintain the quality and consistency of the ingredients, the crude extracts were prepared in a single batch with adequate quantity for this study. Dried LBE *L Radix* RGE (100 g) were incubated with 900 mL deionized water at 100℃ for 2 h. The herbal juice was then centrifuged at 9000 r/min for 20 min to remove the precipitate. The remaining herbal juice was filtered with gauze. The filtered supernatant containing polysaccharides was precipitated with three volumes of 950 mL/L ethanol, concentrated (rotavapor R200 with glass assembly V; BÜCHI Labortechnik AG, Flawil, Switzerland) and lyophilized (freeze dry system Lyph-Lock 6; Labconco Corp., Kansas City, MO, USA)[16].

Composition analysis of herbal extracts

The total carbohydrate content in hot water-extracted LBE and RGE was determined by phenol-sulfuric acid assay using glucose as a standard[17]. The contents of crude protein, crude fat, moisture, and ash were measured using the Association of Official Analytical Chemists (AOAC) methods (981.10, 991.36, 925.10, 923.03)[18].

Cell cultures and treatments

Rat (H-4-II-E, BCRC no. 60209) and human HCC (HA22T/VGH, BCRC no. 60168) cell lines were purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Rat and human HCC cells (1×10^5 cells/mL) were grown in 850 mL/L minimum essential medium (MEM; GIBCO™, Invitrogen Corp., Carlsbad, CA, USA) with 150 mL/L fetal bovine serum (FBS; GIBCO™) or 900 mL/L Dulbecco's modified Eagle's medium (DMEM; GIBCO™) with 100 mL/L FBS, respectively, at 37℃ in a humidified atmosphere of 950 mL/L air and 50 mL/L CO_2. Prior to addition of the treatment, the cells were grown to 80%-90% confluency and synchronized by incubating in serum-free basal medium (MEM or DMEM) for 24 h. The cells were then treated with various concentrations of crude LBE (0-5 g/L) or RGE (0-10 g/L) in the absence of serum for 0-24 h. The cells and medium were collected. Protein contents in the cells and medium were determined by the modified method of Lowry *et al*[19] using a Bio-Rad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA).

Cell proliferation assay

Cell proliferation was colorimetrically measured at 490 nm using a commercial proliferation assay kit (CellTiter 96® AQ_{ueous}; Promega Corp., Madison, WI, USA). After treatment with various concentrations of crude LBE (0-5 g/L) or RGE (0-10 g/L) for 0-24 h, rat and human HCC cells (n = 6) in the 96-well plate were incubated with 20 μL MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution containing 1.90 g/L MTS and 300 μmol/L phenazine methosulfate in Dulbecco's PBS (pH 6.0) for 2 h at 37℃ in a humidified 50 mL/L CO_2 atmosphere[20]. The absorbance of soluble formazan produced by cellular reduction of MTS was determined at 490 nm using an ELISA reader (Multiskan RC; Labsystems, Helsinki, Finland).

Flow cytometric analysis of cellular DNA content

The percentage of cells undergoing apoptosis and distributing in different phases of cell cycle were determined by propidium iodide (PI)-staining method using flow cytometry. After treatment for 24 h, the conditioned medium of rat H-4-II-E cells (n = 6) was centrifuged at 800 r/min for 5 min at 4℃ to remove the supernatant. The trypsinized cells and cell pellet of the conditioned medium were washed with PBS, fixed in 2 mL of 700 mL/L cold ethanol, and stored at 4℃ overnight. After washed twice with PBS, the ethanol-fixed cells were incubated with 3 μL RNase (10 g/L) at 37℃ for 30 min, and stained with 1 mL PI (40 mg/L) in the dark. The cell suspension was then filtered through a 35 μm mesh, and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, CA, USA) within 2 h. Cellular DNA content was calculated using CellQuest software (Becton Dickinson Biosciences).

Analysis of p53 protein

After incubation with various concentrations of LBE (0-5 g/L) or RGE (0-10 g/L) for 24 h, the conditioned medium of rat H-4-II-E cells was centrifuged at 800 r/min for 5 min at 4℃ to remove the supernatant. Rat H-4-II-E cells and cell pellet in the conditioned medium were re-suspended in lysis buffer (2 mol/L Tris, 5 mol/L NaCl, 50 g/L NP-40, 100 g/L sodium dodecylsulfate (SDS), and 5 g/L phenylmethylsulfonyl fluoride), centrifuged at 300 r/min for 5 min at 4℃. The supernatant (40 μg total protein) pooled from 3 independent experiments (n = 3) was mixed with 4 × sample buffer (0.25 mol/L Tris-HCl, pH 6.8, 80 g/L SDS, 200 ml/L glycerol, 100 g/L β-mercaptoethanol, and 1 g bromophenol blue)[21] denatured at 100℃ for 3 min, and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Mini-PROTEAN 3 Cell; Bio-Rad Laboratories). Proteins were separated by 125 mL/L resolving gel (acrylamide: bisacrylamide = 37.5:1) with 40 mL/L stacking gel in the running buffer (25 mmol/L Tris, pH 8.3, 192 mmol/L glycine, and 1 g/L SDS) at 3 EV/cm per hour. Then the proteins were transferred onto nitrocellulose membrane (0.45 μm) using a semi-dry transfer unit (Hoefer Semiphor

Table 1 Composition of hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa*[1]

Ingredient (mg/g)	*Lycium barbarum* extract	*Rehmannia glutinosa* extract
Carbohydrate	762.8	700.4
Protein	134.7	47.2
Fat	3.9	2.9
Moisture	221.6	92.4
Ash	0.5	0.4

[1]The samples were at least triplicated.

TE 70, Amersham Biosciences Corp., San Francisco, CA, USA) in Towbin buffer (25 mmol/L Tris, 192 mmol/L glycine, 1 g/L SDS, and 100 mL/L methanol) at 6 mA/cm per hour[22]. The membrane was washed with PBS, and incubated with a blocking buffer (50 g/L skim milk and 2 mL/L Tween-20 in PBS) for 2 h. Then the membrane was incubated with an anti-mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against p53 (Pab-246; 2 mg/L) or α-tubulin (TU-02; 1 mg/L) at room temperature for 2 h. Alpha-tubulin was used as an internal control. The membrane was washed three times with the wash buffer (2 mL/L Tween-20 in PBS), and incubated with 4 mg/L goat anti-mouse IgG-horseradish peroxide conjugate (Santa Cruz Biotechnology, Inc.) for 2 h. The blot was washed three times again with the wash buffer, incubated with luminol reagent (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) for 2 min, and exposed to an X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 3-5 min. The bands were quantitated by an image analysis system (0.2-megapixel charge-coupled device camera, gel analysis system; EverGene Biotechnology, Taipei, Taiwan) and Phoretix 1D Lite software (version 4.0; Phoretix International Ltd., Newcastle upon Tyne, UK).

Statistical analysis

Data are expressed as mean ± SD. Data were analyzed by one- and two-way analysis of variance (ANOVA) to determine the treatment effect using the Statistical Analysis System (SAS version 8.2; SAS Institute Inc., Cary, NC, USA). Fisher's least significant difference test was used to make *post hoc* comparisons if the treatment effect was demonstrated. $P < 0.05$ was considered statistically significant.

RESULTS

Composition analysis of herbal extracts

The extraction rate for crude LBE and RGE was 704 mg/g and 724 mg/g, respectively. Carbohydrate content, the major component, was 762.8 mg/g and 700.4 mg/g in crude LBE and RGE, respectively (Table 1). Moisture and protein contents in crude LBE and RGE were 221.6 mg/g and 134.7 mg/g as well as 92.4 mg/g and 47.2 mg/g, respectively. Fat and ash contents in these crude extracts were less than 5 mg/g.

Cell proliferation assay

Crude LBE and RGE at a dose of 1 g/L did not inhibit cell proliferation up to 24 h incubation in rat H-4-II-E cells (Table 2). However, crude RGE at a dose of 1 g/L slightly increased cell proliferation of H-4-II-E cells at 18 h incubation compared with the control group (111.4% ± 11.7% *vs* 100.0% ± 5.9% of control, $P = 0.03 < 0.05$). Crude LBE (2-5 g/L) and RGE (2-10 g/L) dose-dependently inhibited cell proliferation by 11% ($P < 0.05$) to 85% ($P < 0.01$) compared with the control group after 6-24 h incubation. The inhibitory effect of crude LBE and RGE at lower doses (1-2 g/L) on cell proliferation was not significantly different. A higher dose (5 g/L) of crude LBE suppressed cell proliferation more efficiently ($P < 0.01$) than that of crude RGE. From the curves of four time points, the mean values of IC50 for crude LBE and RGE were 3.8 g/L and 6.9 g/L, respectively. Similar results in human HA22T/VGH cells, crude LBE (2-10 g/L) and RGE (2-5 g/L) dose-dependently inhibited cell proliferation by 14%-43% ($P < 0.01$) compared with the control group after 24 h incubation (Table 3). Cell proliferation was inhibited equivalently by crude LBE and RGE at lower doses (2-5 g/L). However, crude LBE at a higher dose (10 g/L) showed better suppression (56.8% ± 1.6% *vs* 70.3% ± 3.1% of control, $P = 0.0003 < 0.01$) on cell proliferation compared with the same dosage of crude RGE. The IC50 value for both crude LBE and RGE was above 10 g/L.

Table 2 Effect of hot water-extracted *Lycium barbarum* (LBE) and *Rehmannia glutinosa* (RGE) on cell proliferation in rat hepatocellular carcinoma (H-4-II-E) cells ($n = 6$, mean ± SD)

	Cell proliferation (% of control)			
	6 h	12 h	18 h	24 h
Control	$100.0 \pm 0.7^{e,h,j}$	$100.0 \pm 8.6^{e,h,j}$	$100.0 \pm 5.9^{f,h,j}$	$100.0 \pm 5.9^{f,h,j}$
1 g/L LBE	$100.3 \pm 9.8^{f,h}$	$100.7 \pm 8.2^{e,h}$	100.1 ± 5.7^{f}h	$97.8 \pm 6.3^{f,h}$
2 g/L LBE	$84.4 \pm 6.6^{a,d,h}$	$89.3 \pm 7.5^{a,c,h}$	$83.8 \pm 7.5^{b,d,h}$	$86.3 \pm 2.9^{b,d,h}$
5 g/L LBE	$37.6 \pm 6.9^{b,d,f}$	$23.8 \pm 4.6^{b,d,f}$	$18.0 \pm 5.3^{b,d,f}$	$15.1 \pm 3.1^{b,d,f}$
1 g/L RGE	$99.5 \pm 7.1^{f,h,j}$	$97.0 \pm 13.4^{h,j}$	$111.4 \pm 11.7^{a,f,h,j}$	$96.1 \pm 5.3^{f,h,j}$
2 g/L RGE	$80.9 \pm 18.4^{b,d,h,j}$	$88.3 \pm 9.4^{a,h,j}$	$84.6 \pm 14.4^{b,d,h,j}$	$84.4 \pm 6.4^{b,d,h,j}$
5 g/L RGE	$58.3 \pm 11.4^{b,d,f,j}$	$74.6 \pm 7.0^{b,d,f,j}$	$57.1 \pm 10.1^{b,d,f,j}$	$71.2 \pm 6.7^{b,d,f,j}$
10 g/L RGE	$29.2 \pm 6.9^{b,d,f,h}$	$29.5 \pm 6.3^{b,d,f,h}$	$25.0 \pm 5.7^{b,d,f,h}$	$24.2 \pm 7.1^{b,d,f,h}$

$^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* 1 g/L corresponding treatment, $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* 2 g/L corresponding treatment; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* 5 g/L corresponding treatment, $^{j}P < 0.01$ *vs* 10 g/L corresponding treatment within the same column.

Table 3 Effect of hot water-extracted LBE and RGE on cell proliferation in human hepatocellular carcinoma (HA22T/VGH) cells after 24 h incubation ($n = 6$, mean ± SD)

	Cell proliferation (% of control)
Control	$100.0 \pm 4.2^{d,f,h}$
2 g/L LBE	$86.0 \pm 5.9^{b,c,h}$
5 g/L LBE	$77.0 \pm 5.1^{a,b,h}$
10 g/L LBE	$56.8 \pm 3.9^{b,d,f}$
2 g/L RGE	$86.3 \pm 5.9^{b,f,h}$
5 g/L RGE	$74.3 \pm 7.5^{b,d}$
10 g/L RGE	$70.3 \pm 7.7^{b,d}$

$^{b}P < 0.01$ *vs* control; $^{a}P < 0.05$, $^{d}P < 0.01$ *vs* 2 g/L corresponding treatment; $^{c}P < 0.05$, $^{f}P < 0.01$ *vs* 5 g/L corresponding treatment; $^{h}P < 0.01$ *vs* 10 g/L corresponding treatment.

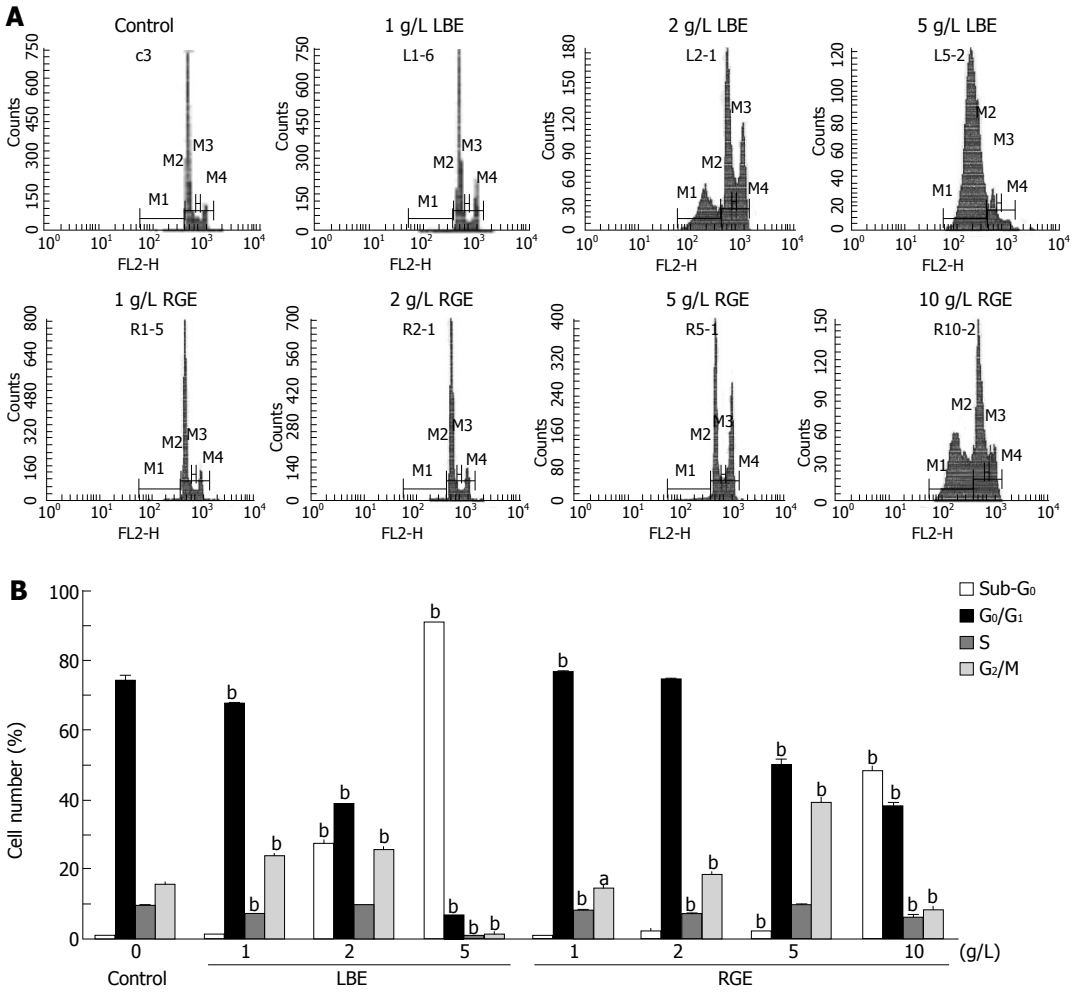

Figure 1 Representative DNA histograms (**A**) and percentage of cells in different cell cycle phases (**B**) after incubated with various concentrations of hot water-extracted LBE and RGE for 24 h in rat H-4-II-E cells determined by flow cytometry. Values are mean ± SD (n = 6). (□), sub-G_0 phase (M1 peak); (■), G_0/G_1 phase (M2 peak); (▩), S phase (M3 peak); (▣), G_2/M phase (M4 peak), $^aP < 0.05$, $^bP < 0.01$ *vs* control within the same cell cycle phase.

Flow cytometric analysis of cellular DNA content

Cellular DNA content in cell cycle distribution was determined, and apoptosis was quantitated by the percentage of cells with sub-G_0 DNA content by flow cytometry (Figure 1A). Crude LBE and RGE at the doses above 2 g/L and 5 g/L, respectively, significantly increased the percentage of cells in sub-G_0 phase (27.5% ± 0.5% and 91.2% ± 0.3% for 2 g/L and 5 g/L LBE, and 2.4% ± 0.2% and 48.1% ± 2.0% for 5 g/L and 10 g/L RGE, $P < 0.01$) compared with the control group (1.0% ± 0.2%) (Figure 1B). The percentage of cells in G_0/G_1 phase was dose-dependently decreased by crude LBE and RGE. Crude LBE at lower doses (1-2 g/L) significantly increased the percentage of cells in G_2/M phase (24.1% ± 0.4% and 25.8% ± 0.3%, $P < 0.01$) compared with the control group (15.8% ± 0.4%), but decreased the percentage of cells to 1.5% ± 0.1% ($P < 0.01$) at a higher dose (5 g/L). Likewise, crude RGE at lower doses (2 g/L and 5 g/L) significantly increased the percentage of cells in G_2/M phase (18.3% ± 0.4% and 39.3% ± 2.1%, $P < 0.01$), but decreased the percentage of cells to 8.3% ± 0.7% ($P < 0.01$) at a higher dose (10 g/L).

Analysis of p53 protein

After 24 h incubation with crude LBE and RGE, the expression of p53 protein in rat H-4-II-E cells was analyzed by SDS-PAGE and Western blotting (Figure 2A). After calibrated by an internal control (α-tubulin), the expression of p53 protein increased with the dosage of crude LBE and RGE (Figure 2B). Crude LBE at higher doses (2-5 g/L) increased p53 expression to 119% and 143% of the

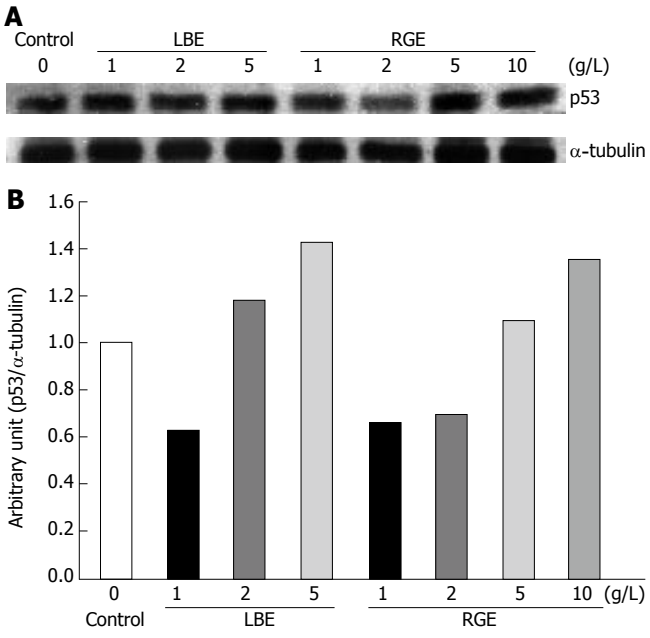

Figure 2 Expression of p53 protein with the molecular weight of 53 ku visualized by Western blotting (**A**) and quantitated by an image analysis system (**B**) after incubation of rat H-4-II-E cells with various concentrations of hot water-extracted LBE and RGE for 24 h. Samples were pooled from 3 independent experiments (n = 3). Alpha-tubulin (55 ku) was used as an internal control.

control group, respectively. Similarly, crude RGE at higher doses (5-10 g/L) increased p53 expression to 110% and 136% of the control group, respectively. However, lower doses of crude LBE (1 g/L) and RGE (1-2 g/L) suppressed p53 expression to 63%-70% of the control group.

DISCUSSION

Polysaccharides, a water-soluble bioactive component from LBE *L Radix* RGE, showed antitumorgenic activity[3,15]. The methods for the isolation of polysaccharides from these herbs are various in different studies. Different extraction methods and fractions could affect not only the ingredients but also the physiological activity of the extract. Our crude herbal extracts were prepared by hot water (100℃) extraction followed by ethanol (950 mL/L) precipitation. Gan *et al*[3] extracted LBE with distilled water at 80℃ after removal of pigment by acetone/petroleum (1:1) and oligosaccharides by 800 mL/L ethanol. The crude polysaccharide-protein complex was precipitated by ethanol, and the acidic fraction was further purified by diethylaminoethyl-cellulose anion exchange and Sephadex G200 column chromatography. Finally, a purified fraction (LBP_{3p}) contains 63.6% neutral sugars, 24.8% acidic sugars, and 7.6% proteins. The purified fraction showed antiproliferating and immunomodulating activity in S180-bearing mice. Compared with the purified LBP_{3p} fraction, our crude LBE contains lower sugars (88.4% *vs* 76.3%) and higher proteins (7.6% *vs* 13.5%). Zhang *et al*[4] prepared purified fraction of RGE oligosaccharides by extraction with hot water and isolation with cation/anion exchange and charcoal column chromatography. The eluted fraction containing monosaccharides, disaccharides, trisaccharides, and other oligosaccharides but not polysaccharides exert a significant hypoglycemic effect in normal and alloxan-induced diabetic rats. Our crude extract did not contain certain water-insoluble active components in LBE and RGE. Scopoletin (7-hydroxy-6-methoxycoumarin), slightly soluble in water, could be excluded as extracted by hot water, and has been reported as an active component of the fruit of LBE for inhibiting cell proliferation of human prostate cancer PC3 cells[23]. Furan derivatives, isolated from chloroform extract of the dried roots of RGE, have the immunomodulating and anti-coagulating activity[24].

Our study found that both crude LBE and RGE suppressed cell proliferation in rat and human HCC cells in a dose-dependent manner. Consistent with our findings, Zhang *et al*[25] showed that polysaccharide containing LBE extract inhibits cell proliferation of human hepatoma QGY7703 cells. Additionally, LBE polysaccharides (20-1000 mg/L) dose-dependently suppress cell growth of human leukemia HL-60 cells[26]. The previous *in vivo* studies also showed that LBE exhibits the antitumorgenic activity in tumor-bearing mice[3,27]. A polysaccharide-protein complex from LBE (LBP_{3p}) at 10 μg/g significantly reduces tumor weight and enhances immune functions in S180-bearing mice[3]. RGE polysaccharide b with an average molecular weight of 160 ku has antitumorgenic and immunomodulating activity in S180-bearing mice[13]. A clinical trial found 40.9% response rate in the advanced cancer patients with lymphokine-activated killer (LAK) cells and interleukin-2 (IL-2) treatment combined with LBE polysaccharides compared with 16.1% response rate ($P < 0.05$) in those treated with LAK/IL-2 alone. These data indicate that LBE polysaccharides can be used as an adjuvant for the treatment of cancer[28]. Chinese medicinal herbs containing RGE have been demonstrated to alleviate the adverse effects of high-dose methotrexate plus vincristine in post-operative osteogenic sarcoma patients with chemotherapy, suggesting that Chinese medicinal herbs containing RGE are less toxic compared with a traditional chemotherapy[29]. The extract of LBE at the dose of 25 g/L and 5 g/L inhibited cell proliferation by 14% and 85%, and stimulated p53 protein expression by 19% and 43% in rat H-4-II-E cells after 24 h incubation. The extract of RGE at the dose of 5 g/L and 10 g/L inhibited cell proliferation by 23% and 76%, and stimulated p53 protein expression by only 10% and 36% in rat H-4-II-E cells after 24 h incubation. The inhibition of cell proliferation by LBE at the dose of 2 g/L was almost parallel to the stimulation of p53. However, the relationship between cell proliferation inhibition and p53 expression was not obvious in rat H-4-II-E cells treated with higher doses of LBE and RGE.

The percentage of cells in sub-G_0 phase significantly increased in H-4-II-E cells after 24 h treatment with higher doses of crude LBE (2-5 g/L) and RGE (5-10 g/L). A dramatic increased cell percentage in sub-G_0 phase by crude LBE and RGE at higher doses was probably due to late apoptotic/necrotic cells in sub-G_0 phase if undergoing autolysis, which could overestimate the apoptotic cells. The proliferation-inhibiting and apoptosis-inducing activity of crude LBE at higher doses (≥ 5 g/L) in HCC cells was more effective than that of crude RGE according to the mean values of IC50 and the proportion of apoptotic cells. As initiating a significant increase in the percentage of cells in sub-G_0 phase by crude LBE (2 g/L) and RGE (5 g/L), the percentage of cells in G_2/M phase significantly increased. Additionally, the expression of p53 protein was correspondently stimulated as well after 24 h incubation with 2 g/L crude LBE and 5 g/L crude RGE. However, lower doses of crude LBE and RGE decreased the expression of p53 protein compared with the control group, which could be due to the overexposure to α-tubulin. The expression of p53 protein tended to be dose-dependently increased by crude LBE and RGB. The results suggest that higher doses of crude LBE and RGE arrest cells in G_2/M phase and p53 may be involved in mediating apoptosis. The mechanism of promoting G_2/M arrest by LBE and RGE has not been understood. It is proposed that LBE and RGE may inhibit nuclear factor-κB to alter the expression of regulatory cell cycle proteins such as cyclin B and/or p21WAF1/Cip1. A previous study reported that LBE polysaccharides arrest cells in S phase and induce apoptosis with increased intracellular calcium in human hepatoma QGY7703 cells[25]. Additionally, LBE (20-1000 mg/L) results in DNA fragmentation and positive TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) signals in human leukemia HL-60 cells, indicating that LBE induces apoptosis[26]. Although the effect of LBE and RGE on apoptosis in normal hepatocytes has not been studied yet, the previous studies[30,31] found

that LBE protects normal cells from apoptosis rather than stimulating apoptosis in tumor cells. LBE dose-dependently inhibits apoptosis induced by hydrocortisone in rat spleen *in vitro*[30]. LBE extract shows cytoprotective effect against β-amyloid peptide-induced apoptosis in primary rat cortical neurons and dithiothreitol-induced caspase-3 activation in normal neural endoplasmic reticulum[31]. The effect of LBE on apoptosis could be various due to different extraction methods, extraction fractions, dosages, tissues, and cell types (normal *vs* malignant). The low-molecular-weight RGE polysaccharides at the doses of 20 μg/g and 40 μg/g markedly increase the level of p53 mRNA to 3.3- and 3.2-fold, respectively, in Lewis lung cancer tissue of C57BL/6 mice[15]. Activation of p53 tumor suppressor protein can lead to cell cycle arrest and apoptosis in response to DNA damage[32]. Cell death induced through p53-mediating pathway is subsequently initiated by the activation of caspases followed by the characteristic apoptotic phenotype.

The mechanisms underlying regulation of apoptosis by LBE and RGE have not been clearly understood. Besides the induction of p53-mediated apoptosis, immunomodulation may contribute to antitumorgenesis. A polysaccharide-protein complex from LBE (LBP_{3p}) dose-dependently increases the expression of IL-2 and tumor necrosis factor (TNF)-α at the levels of mRNA and protein in human peripheral blood mononuclear cells[33]. An *in vivo* study showed that LBP_{3p} administered orally at 10 μg/g for 10 d inhibits cell growth of transplantable sarcoma S180 cells as well as increases macrophage phagocytosis, cell proliferation of spleen lymphocytes, the activity of cytotoxic T lymphocytes (CTL), and the expression of IL-2 mRNA in S180-bearing mice[3]. RGE polysaccaride b at the intraperitoneal injection dose of 10 μg/g or 20 μg/g attenuates the decrease in CTL activity caused by excessive tumor growth through increasing the production of $CD8^+$ ($Lyt\text{-}2^+$) CTL and lowering the ratio of $CD4^+$ to $CD8^+$ ($L3T4^+$) T lymphocyte subset in S180-bearing mice[13]. An aqueous extract of RGE-steamed root dose-dependently suppresses the secretion of TNF-α by mouse astrocytes stimulated with substance P and lipopolysaccharide through the inhibition of IL-1 secretion, suggesting that RGE has the anti-inflammatory activity[14]. Although crude LBE and RGE show antitumorgenic activity *in vitro*, it still remains to determine the antitumorgenic components of crude LBE and RGE, and molecular mechanisms for regulating the proliferation of HCC cells.

In conclusion, hot water-extracted LBE (2-5 g/L) and RGE (5-10 g/L) inhibit proliferation and stimulate apoptosis probably involved in p53 mediation in HCC cells. It is not known that whether hot water-extracted LBE and RGE have antiproliferative and apoptosis-stimulating effects on HCC *in vivo* and further studies are still required to verify their *in vivo* effects on both normal and malignant hepatocytes.

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Risk of colon cancer in hereditary non-polyposis colorectal cancer patients as predicted by fuzzy modeling: Influence of smoking

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Abstract

AIM: To investigate whether a fuzzy logic model could predict colorectal cancer (CRC) risk engendered by smoking in hereditary non-polyposis colorectal cancer (HNPCC) patients.

METHODS: Three hundred and forty HNPCC mismatch repair (MMR) mutation carriers from the Creighton University Hereditary Cancer Institute Registry were selected for modeling. Age-dependent curves were generated to elucidate the joint effects between gene mutation (hMLH1 or hMSH2), gender, and smoking status on the probability of developing CRC.

RESULTS: Smoking significantly increased CRC risk in male hMSH2 mutation carriers ($P < 0.05$). hMLH1 mutations augmented CRC risk relative to hMSH2 mutation carriers for males ($P < 0.05$). Males had a significantly higher risk of CRC than females for hMLH1 non smokers ($P < 0.05$), hMLH1 smokers ($P < 0.1$) and hMSH2 smokers ($P < 0.1$). Smoking promoted CRC in a dose-dependent manner in hMSH2 in males ($P < 0.05$). Females with hMSH2 mutations and both sexes with the hMLH1 groups only demonstrated a smoking effect after an extensive smoking history ($P < 0.05$).

CONCLUSION: CRC promotion by smoking in HNPCC patients is dependent on gene mutation, gender and age. These data demonstrate that fuzzy modeling may enable formulation of clinical risk scores, thereby allowing individualization of CRC prevention strategies.

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Key words: Hereditary non-polyposis colorectal cancer; Lynch syndrome; Smoking; Colorectal cancer; Fuzzy modeling; Risk assessment

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INTRODUCTION

Accurate risk-stratification is essential for combating the 50 000 yearly deaths from colorectal cancer (CRC) in the United States[1]. The best-established risk factor is a familial predisposition to CRC, which is implicated in one-quarter of all CRC cases[2]. While determining a family history can be readily accomplished, risk quantification which is critical for tailoring screening strategies, remains remarkably imprecise. For instance, even in documented carriers of CRC predisposing genes, clinical expression can be quite varied due to modulation by numerous confounding endogenous and exogenous variables[3,4].

Hereditary non-polyposis colorectal cancer (HNPCC) represents a case in point. This autosomal dominant condition is the most common cancer predisposing syndrome engendering a > 70% lifetime risk of developing CRC[5]. Furthermore, we have recently demonstrated that cigarette smoking of a male carrying hMLH1 mutations (versus hMSH2) increases the hazard of CRC by 1.4-, 1.6- and 2.0-fold respectively[6]. However, optimal management strategies (colonoscopic surveillance versus prophylactic colectomy) are unclear secondary to characteristic phenotypic heterogeneity, i.e. marked variations in age of onset of cancers[7]. Thus, HNPCC represents an excellent paradigm to study the gene-environment joint effect hypothesis.

Incorporating these important findings into clinical

practice is hindered by the inability to accurately quantitate the risk modulation engendered by the joint effects of genetics and environmental factors. Moreover, the inadequacy of conventional statistical approaches to model the complex nature of many of the CRC risk factors further limits application of these data to patient management. While landmark studies have explored the age of CRC diagnosis in HNPCC[8], these estimates along with others in the literature have not yet factored in the genetic/environmental influences that determine the phenotypic heterogeneity. The focus of our study was on this phenotypic heterogeneity which is of major importance to clinicians who care for these challenging patients. We believe that by knowing the genetic and environmental risk factors, we can individualize more accurately the risk analysis, which, to our knowledge, has not been previously reported.

One approach from the engineering literature that has recently received attention for cancer risk assessment is fuzzy logic. This powerful modeling technique has been successfully used for pattern recognition and image processing and its unique ability to transcend the typical black or white approaches in standard modeling and to capture the "shades of gray" has great promise for clinical medicine[9]. While typical statistical approaches function well when the data are normally distributed and values are near the mean, this approach is often inadequate at the threshold. For instance, a very high prostate specific antigen (PSA) has excellent predictive ability for prostate cancer, but the optimal clinical management of a patient with a mildly elevated value is unclear. Fuzzy logic overcomes these limitations of conventional statistics by allowing partial membership function. In our PSA example, instead of categorizing values as either normal or abnormal, a fuzzy approach would allow one to place a value as one quarter in the normal group and three-quarters in the abnormal group. Thus, through the creation of fuzzy sets, elements can have degrees of membership on a continuum (e.g. a value can be "normal, slightly elevated, moderately elevated or highly elevated").

Another unique attribute of fuzzy modeling is that, unlike traditional models, it does not require prior knowledge of the system being modeled. It is a "model-free" form in which natural rules are developed from the data rather than imposing rules on the modeling system. The result of this "model-free" system is still a conversion from inputs to outputs, similar to traditional algorithms[10]. Another strength of fuzzy modeling lies in its ability to model data points that may be outside of traditional inclusion boundaries and thus allowing accurate modeling with less data.

Past reports have demonstrated that fuzzy modeling can improve performance characteristics of tumor markers over conventional applications[11-13]. Our previous work with conventional statistical approaches (COX proportional hazard modeling) indicated that tobacco use, gender and mutated gene play an important role in phenotypic presentation of CRC progression in HNPCC patients as a group, but lack the ability to predict an individual risk of CRC (e.g. not sensitive to dose effects or interactions of factors). We, therefore, explored the ability of fuzzy modeling to predict CRC risk in germline mutation carriers in these individual HNPCC patients by factoring the gene type, gender and tobacco use status in the present study.

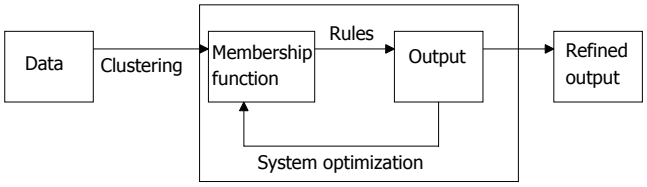

Figure 1 Overview of fuzzy methodology.

MATERIALS AND METHODS

Database

The Hereditary Cancer Center at Creighton University is one of the oldest and largest registries for diverse hereditary cancer syndrome, containing information on over 200 000 individuals of whom approximately 600 are verified MMR mutation carriers (Lynch Database). The database contains patient and family information, surveillance and treatment information as well as gene mutations and lifestyle data. The inclusion criteria were HNPCC as documented by either a MMR germline mutation positivity or clinical HNPCC from a patient who had a family member with a documented MMR mutation. For example, if the patient has HNPCC and his mother has a documented hMSH2 mutation, we would consider the patient to have an hMSH2 mutation. Tobacco data were obtained by self-report and family report or by abstraction from medical records. A patient was classified as a tobacco user if he/she reported ever regularly using (or was reported to have ever regularly used) cigarettes, cigars, a pipe, tobacco chew, or snuff. Five hundred and ninety-six mutation carriers were identified from 62 HNPCC families. For this analysis we only focused on cigarette smokers. Of these, 340 (60.4%) had information on tobacco use and were included in our study (158 nonsmokers and 182 smokers). In a further analysis, 271 patients (113 of 182 smokers and 158 nonsmokers) with a more detailed smoking history including calculated pack-years were selected.

Modeling

The entire modeling procedure was performed using the MatLab Fuzzy Toolbox (Matlab, Version 6.1-Release 13, Natick, MA). Figure 1 provides an overview of the basic fuzzy modeling algorithm. The data (either categorical or continuous) were inputted into the program and a clustering algorithm led to the development of membership functions. The fuzzy clustering method used produces descriptions of each of the input vectors as belonging to one (or more) fuzzy sets with a specific membership in each of the sets. The inclusion of a continuous variable (pack-years and age) produces non-catagorical (aka, fuzzy) memberships. Rules are then developed from these membership functions which successfully produce a mapping from the input space to the output space as previously described[10]. Furthermore,

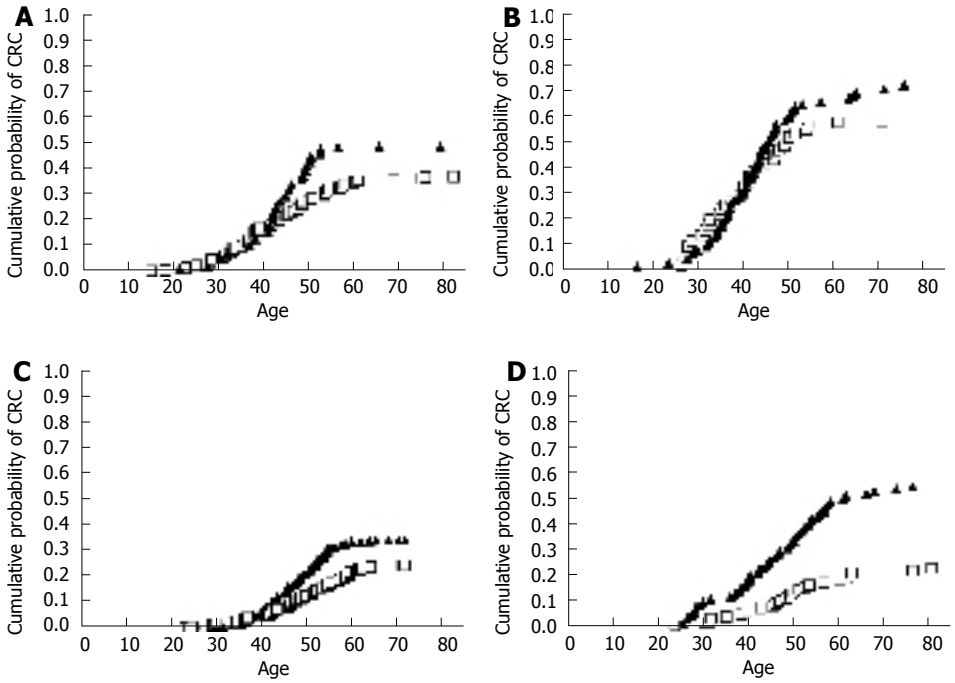

Figure 2 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on smoking status, ▲ = Smokers, □ = Non-Smokers. Data are divided by gene mutation and sex with (**A**) hMLH1: Female, (**B**) hMLH1: Male, (**C**) hMSH2: Female and (**D**) hMSH2: Male ($P < 0.05$).

these rules represent fuzzy relationships between the variables, even if the variables themselves are categorical. The refinement of these rules is accomplished using the Adaptive Neural Fuzzy Inference System (ANFIS; 16) which acts as a feedback loop to further refine the rules until they are optimized to give the best fit to the data. Overfitting of the model to the data is not exclusively addressed. However, the clustering methods (subtractive clustering) employed in the modeling scheme tend to partition the data space in such a manner as to maximize the cluster density while simultaneously maximizing the separation of the clusters which would limit overfitting.

This modeling technique was applied to the patients selected from the Lynch database with gene mutation, sex, smoking status and age as the input and risk of developing colorectal cancer as the output. In a second study, the effect of pack years was added as an additional input. In this case, dividing a group of smokers with a given mutation, sex and age further by smoke years made the numbers in each group quite small. We therefore used the data as the training set and a theoretical set of conditions as the input to generate the model output. The models produced a cumulative risk of CRC that ranged between 0 and 1. Age and pack years were fuzzified in the program.

Statistical analysis

Results are presented either as a scattergram of the actual model output for each patient in the database (Figures 2-4) or as an output of the model given a set of theoretical conditions (Figure 5). The statistical procedures used followed the methods described by Steel and Torrie[14]. The data for each cohort were paired and compared using a Kolmagrov-Smirnov (KS) 2-sample test. The KS test was considered to be conservative and useful when hypothesis about discrete distributions was tested. The test is motivated by the need to compare 2 independent samples and the null hypothesis is that each sample originates from identical distributions (i.e., the data are from the same population). Critical values for the KS test are inversely proportional to the square root of the total number of observations. The nature of our data dictated that the critical values were computed using unequal populations ($n_1 \neq n_2$). Furthermore, the nature of the data and results only required a comparison of this type. Other analyses, such as a test of trend or analysis of variance, were not deemed beneficial.

RESULTS

Smoking status

The influence of genetic mutation combined with sex, smoking status and age is demonstrated in Figure 2. There was a clear impact of cigarette smoking on the age-adjusted risk of developing CRC for all conditions tested (gene mutated and gender).

When a male patient with the hMSH2 gene mutation carrier smokes, he markedly increased his risk of developing CRC by up to 2.4-fold at the age of 78 (Figure 2D, $P < 0.05$). In the case of a mutation in the hMLH1 gene, smoking increased the risk of CRC at the maximal age tested by approximately 1.3-fold for males when compared to non-smokers (Figure 2B). Females with the hMLH1 mutation showed a 1.3-fold increased risk of developing CRC and female smokers with the hMSH2 mutation had a 1.4-fold greater risk of developing CRC when compared to their non-smoking age-matched controls (Figures 2A and 2C).

Gene

The model output was then reexamined as a function of the genetic mutation (Figure 3). For males, patients with a hMLH1 mutation had either a 2.5- or a 1.3-fold greater risk of developing CRC than those with a hMSH2 for non-smokers and smokers respectively (Figures 3B and 3D, $P <$

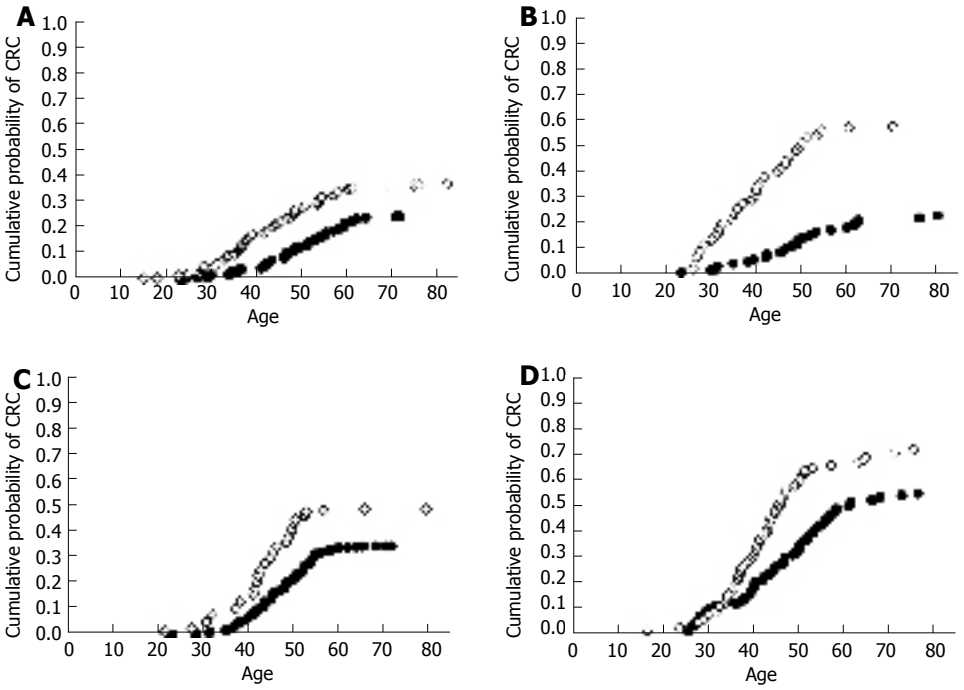

Figure 3 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on genetic mutation, ◊ = hMLH1, ● = hMSH2. Data are divided by sex and smoking status with (**A**) Female: non-smokers ($P < 0.001$), (**B**) Female: smokers ($P < 0.001$), (**C**) Male: non-smokers ($P < 0.001$) and (**D**) Male: smokers ($P < 0.05$). The P values show the probability failing to reject the null hypothesis that the data originate from identical populations, indicating that the curves are different.

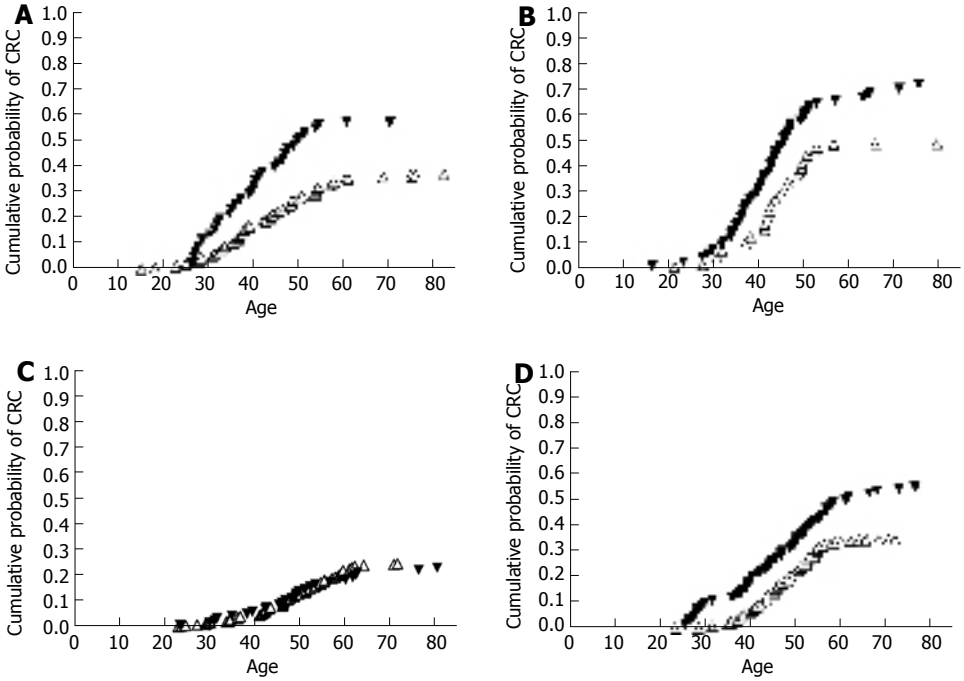

Figure 4 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on gender, △ = female, ▼ =male. Data are divided by gene mutation and smoking status with (**A**) hMLH1: non-smokers, (**B**) hMLH1: smokers ($P < 0.1$), (**C**) hMSH2: non-smokers ($P > 0.05$) and (**D**) hMSH2: smokers ($P < 0.1$). The P values show the probability of failing to reject the null hypothesis that the data originate from identical populations, indicating that the curves are different.

0.05). The difference was greater for the non-smokers than the smokers because subjects with hMSH2 who smoke increased their rate of CRC greater than the non-smokers. Female non-smokers with the hMLH1 mutation showed a 1.5-fold increase in CRC risk as compared to the hMSH2 subjects, whereas it was 1.4-fold higher for smokers.

Gender

Figure 4 demonstrates that males had a greater risk of developing CRC than females when compared to an equivalent age, gene mutation and smoking status. Males had a significantly higher risk of CRC than females (1.5-fold) for non-smoking subjects with the hMLH1 mutations (Figure 4A, $P < 0.05$) and smokers (1.6-fold) (Figure 4B, $P < 0.1$). Smoking males with the hMSH2 mutation also had a greater risk of CRC than hMSH2 mutation harboring females by 1.6-fold (Figure 4D, $P < 0.01$), but the gender effect dissipated in nonsmokers (Figure 4C, $P > 0.05$).

Smoking dose response

A subset of the data which had an estimate of pack years was then modeled to determine if there was an association between lifetime quantities of cigarettes smoked and risk of CRC. The mean consumption was 24-pack years for patients who smoked cigarettes. Figure 5 demonstrates the

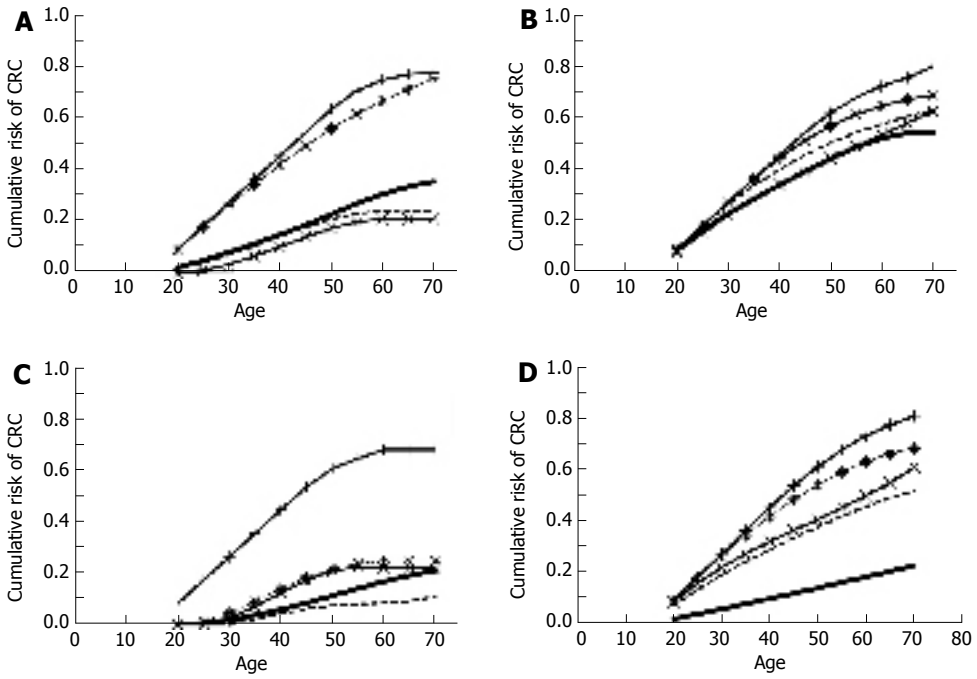

Figure 5 Probability of developing CRC (cumulative lifetime risk) based on a model containing pack years for (**A**) females with hMLH1 mutation ($P < 0.01$), (**B**) males with hMLH1 mutation ($P < 0.05$), (**C**) females with hMLH1 mutation ($P < 0.01$), and (**D**) males with hMSH2 mutation ($P < 0.01$). The *P* values show the probability of failing to reject the null hypothesis that the data originate from identical populations. The number of pack years is as follows: - = 0, -x- = 10, --- = 20, -*- = 30 and -+- = 40.

risk of developing CRC as a function of pack years. For female smokers with a hMLH1 mutation (Figure 5A), the CRC risk was only increased after 30-pack years ($P < 0.01$), whereas for males (Figure 5B) the risk did not significantly increase until 40-pack years ($P < 0.05$). Similarly, females with a hMSH2 (Figure 5C) only demonstrated a smoking effect at 40-pack years ($P < 0.01$). Conversely, males with a hMSH2 mutation (Figure 5D) had an increased risk of developing CRC in a dose-dependent fashion in response to the number of cigarettes smoked over their lifetime ($P < 0.01$).

DISCUSSION

We have demonstrated herein that by using a fuzzy modeling approach, we could quantitatively predict the effect of environmental factors on risk of developing CRC in subjects who harbor a germline mutation for HNPCC. Importantly, we could calculate estimates for the impact of modifiable risk factors (i.e. smoking) on the occurrence of CRC in these high-risk patients and individualize the risk estimates by accounting for other major factors on the phenotypic variability in HNPCC patients: the mutated gene (hMLH1 versus hMSH2) and gender. Thus, we believe that these results may be a useful tool in patient counseling by providing concrete estimates of the impact of risk factor modification.

Our observations regarding the gene-environmental joint effects were made possible by the remarkable resource represented by the Creighton Hereditary Cancer Center Registry. Although it is one of the oldest and largest HNPCC registries in the world, a conventional statistical approach to this dataset is not powerful enough to detect the gene-environment joint effect and the dose-response of smoking and CRC[15], because the relatively small subgroup size markedly reduces statistical power in conventional (e.g. Cox proportional hazard modeling) statistical approaches. One approach to mitigate these concerns is to increase the size of groups (e.g. to evaluate effect of age by increasing 40-59, 60-79, *etc*). However, such large groups have clear disadvantages. For instance, a 41-year old individual and a 58-year old individual may be quite biologically/ clinically different and yet are in the same stratum. Fuzzy modeling enables partial membership functions. For instance, a 43-year old individual may be considered to be 80% in the 40-50 group and 20% belonging to the 30-39 category, whereas a 59-year old individual may be 65% in the 50-59 group and 35% within the 60-70 group. Thus, fuzzy modeling allows us to account for the heterogeneity, i.e., "shades of gray" that is a hallmark of clinical medicine.

There are several lines of evidence that support the biological validity of our findings with fuzzy modeling. Cigarette use is an important risk factor for CRC, and 12% of all CRC deaths are attributed to smoking[16]. Many studies indicate that cigarette smoking can increase the incidence of colon cancers by approximately two fold, however there are numerous contradictory reports[17-19]. These discordant data have been clarified by the demonstration that cigarette smoking may selectively increase the risk for DNA mismatch repair[20]. This may be related to the observation that smoking preferentially promotes microsatellite unstable (MSI-high) tumors. The molecular pathway is also seen in Lynch syndrome tumors[19]. For instance, Yang *et al*[21] have recently reported that cigarette smoking increases the risk of developing MMR-deficient tumors by 3.1-fold. Additionally, Slattery *et al*[22] demonstrated that smoking 20 cigarettes per day increases the risk of MMR-deficient tumors by 1.6-fold in men (95% CI = 1.0-2.5) and 2.2-fold in women (95% CI = 1.4-3.5). Furthermore, they have documented a dose-dependent relationship between smoking and colorectal cancer[22]. This dose-dependence underscores the plausibility of the cigarette-induced CRC risk. While we were able to discern an effect of smoking in our previous

study in HNPCC- cigarette smoking data set with Cox proportional hazard modeling, the lack of dose response raises concerns about the validity of the findings[15]. Using fuzzy modeling we demonstrated a much clearer relationship. For instance, our dose response model predicts that female smokers with a hMLH1 mutation who have at least 30-pack years of smoking will have an increase in the lifetime risk of CRC by 2.2- fold. A 3.3-fold increase is seen for females with an hMSH2 mutation after 40 pack years. Males with a hMSH2 mutation have a more linear increase in their lifetime risk of developing CRC as a function of pack years.

This ability to quantitate an individual's risk is of paramount clinical importance due to the variability in CRC presentation that is characteristic of HNPCC. For instance, some members of a kindred may develop CRC at age 25 and 65 while other members may never develop it. Given this heterogeneity, "one size fits all" approach to management (the current state of the art) is clearly inadequate. Indeed, previous attempts to determine the optimal cancer prevention strategy (prophylactic colectomy versus colonoscopic surveillance) have failed to conclusively demonstrate the superiority of any single approach[7]. Even determining the best colonoscopic intervals is unclear. While our group recommends annual colonoscopy starting at age 25[23], a large number of negative examinations are expensive and have potential complications and may lead to patient complacency. Increasing surveillance intervals is fraught with danger given both the rapid adenoma to carcinoma transition and also the flat nature of the lesions, leading to a higher possibility of lesions being missed on colonoscopy[23]. The consequences of inadequate screening are underscored by the report of Jarvinen and colleagues[24], who noted that over a 15-year observation period, 8.4% of HNPCC patients who did not undergo screening would die of CRC whereas none of those who were in a screening program can succumb to this malignancy. Indeed, in mutation positive subjects, development of CRC occur in 42% of the non-screened but only 18% in patients receiving screening ($P < 0.02$)[24]. Thus, implementation and adherence of a screening regimen are critical in protecting these high-risk patients against CRC.

It needs to be emphasized that the ability of fuzzy modeling to quantitate risk may be of considerable importance in counseling patients. For instance, being able to tell patients that their risk of CRC more than doubles with smoking may be more tangible than stating that smoking is detrimental to ones health, thereby providing a greater impetus for behavior modification. By accurately delineating risk, patients will be able to concretely identify modifiable risk factors and ascertain the impact of their lifestyle changes, thus providing positive reinforcement. In this regard, Halpert and colleagues[25] noted that genetic testing of HNPCC patients may have a profound effect upon motivation for cancer prevention strategies such as colonoscopy. Improved adherence with CRC screening regimens from genetic testing and counseling is also documented by Hadley and associates[26]. The malleability of CRC prevention behaviors in HNPCC patients is further highlighted by Adams and colleagues[27] who documented the effect of socio-economic considerations on age of resection of CRC in HNPCC patients. Thus, we believe that added information obtained by fuzzy modeling may have a dramatic effect on patient behavior and thus outcomes. There are previous demonstrations of the efficacy of fuzzy logic to cancer risk stratification. Fuzzy logic has been used with impressive success to improve the sensitivity of tumor markers in diagnosing cancer[11-13].

There are several limitations of this report that need to be acknowledged. As any modeling, the accuracy of the results is dependent on quality of the data inputted. Many of our patients did not have data to quantitate pack-years. Bias in the database due to patient/family report is possible (e.g. having cancer may influence recollection of tobacco use history). Since tobacco use is not a "standard" risk-factor for CRC, we do not think this will impact the results. With smoking, there is always concern about confounding from "competing causes of mortality"[28]. However we have recently shown that this effect is negligible for the smoking-CRC effect[29]. Finally, while our modeling accurately reflects our database, the algorithms need to be validated in other databases.

In conclusion, a fuzzy modeling approach represents a promising means of predicting the phenotypic heterogeneity in colorectal cancer presentation in HNPCC mutation carriers. The methodology may be an important tool in unraveling the gene-environment joint effects in hereditary cancer syndromes. Furthermore, this may serve as the basis for future paradigms that determine individualized cancer prevention strategies in subjects harboring an inherited risk.

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VIRAL HEPATITIS

Inhibition of hepatitis B virus replication by APOBEC3G *in vitro* and *in vivo*

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Abstract

AIM: To investigate the effect of APOBEC3G mediated antiviral activity against hepatitis B virus (HBV) in cell cultures and replication competent HBV vector-based mouse model.

METHODS: The mammalian hepatoma cells Huh7 and HepG2 were cotransfected with various amounts of CMV-driven expression vector encoding APOBEC3G and replication competent 1.3 fold over-length HBV. Levels of HBsAg and HBeAg in the media of the transfected cells were determined by ELISA. The expression of HBcAg in transfected cells was detected by western blot. HBV DNA and RNA from intracellular core particles were examined by Northern and Southern blot analyses. To assess activity of the APOBEC3G *in vivo*, an HBV vector-based model was used in which APOBEC3G and the HBV vector were co-delivered *via* high-volume tail vein injection. Levels of HBsAg and HBV DNA in the sera of mice as well as HBV core-associated RNA in the liver of mice were determined by ELISA and quantitative PCR analysis respectively.

RESULTS: There was a dose dependent decrease in the levels of intracellular core-associated HBV DNA and extracellular production of HBsAg and HBeAg. The levels of intracellular core-associated viral RNA also decreased, but the expression of HBcAg in transfected cells showed almost no change. Consistent with *in vitro* results, levels of HBsAg in the sera of mice were dramatically decreased. More than 1.5 log10 decrease in levels of serum HBV DNA and liver HBV RNA were observed in the APOBEC3G-treated groups compared with the control groups.

CONCLUSION: These findings indicate that APOBEC3G could suppress HBV replication and antigen expression both *in vivo* and *in vitro*, promising an advance in treatment of HBV infection.

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Key words: APOBEC3G; Hepatitis B virus; Antiviral therapy

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma[1]. HBV replication involves reverse-transcription of a pregenomic RNA (pgRNA) intermediate inside nucleocapsids, which are formed by 180 or 240 core protein subunits. Inside the capsid, the viral polymerase converts pgRNA into minus-strand DNA, which in turn is completed to a double-stranded, relaxed circular DNA molecule[2,3]. This life cycle places HBV into the large family of retroelements, which all require reverse-transcription of an RNA intermediate.

Recently, a cellular defense mechanism targeting a wide range of retroviruses was identified. It was shown that the propagation of HIV-1 strains lacking the accessory protein Vif is suppressed in nonpermissive cells due to expression of the cytidine deaminase APOBEC3G[4,5]. Further studies revealed that A3G induces massive C→U deamination of single stranded retroviral DNA, resulting

in DNA degradation or lethal G→A hypermutation[6-8]. In addition, APOBEC3F (another cytidine deaminase of APOBEC family) is also a potent retroviral restrictor, but its activity, unlike that of APOBEC3G, is partially resistant to HIV-1 Vif and results in retroviral hypermutation. Moreover, APOBEC3F and APOBEC3G appear to be coordinately expressed in a wide range of human tissues and are independently able to inhibit retroviral infection. Thus, APOBEC3F and APOBEC3G are likely to function alongside one another in the provision of an innate immune defense, with APOBEC3F functioning as the major contributor to HIV-1 hypermutation *in vivo*[9]. Interestingly, APOBEC3G can also interfere with the HBV life cycle in cotransfected cells[10,11]. Surprisingly, however, reduced levels of encapsidated viral pgRNA rather than extensive editing were found to be the major contributing factor[10]. APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in transfected HepG2 hepatoma cells[11].

In the present study, we performed a detailed analysis of the inhibition effect of APOBEC3G on hepatitis B virus replication in cell culture and in an HBV vector-based mouse model.

MATERIALS AND METHODS

Plasmid constructs

To construct expression vectors coding for human APOBEC3G (pXFA3G), total RNAs were extracted from peripheral blood mononuclear cells (PBMCs). RT-PCR amplification of human APOBEC3G sequence used forward primer 5'-CGGAATTCAAGCCTCACTT CAGAAACAC-3' and reverse primer 5'- CGAAGCTT TCTGCCTTCCTTAGAGACT G-3'. The PCR product was cloned into EcoRI/HindIII restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its carboxy terminal end (pXF3H). Replication competent wild-type HBV 1.3 fold over-length plasmid (subtype, ayw) has been constructed previously in our laboratory.

Cell culture, transfection and harvesting

HuH-7 cells were grown at 37℃ with 50 mL/L CO_2 in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum (Invitrogen, CA, USA). The cells were plated at a density of 4.5×10^5 cells per well in 6-well plates 18 h prior to transfection. Transfecion of cells was performed with lipofectamine 2000 (Invitrogen, CA, USA) following the user guidelines. On d 3 after transfection, the cells were removed from the culture dish by treatment with trypsin-EDTA, resuspended in culture medium, washed with phosphate-buffered saline, pelleted, and resuspended in 1 mL chilled lysis buffer (140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 50 mmol/L Tris-HCl [pH 8.0]) containing 0.5% Nonidet P-40. Nuclei were removed *via* centrifugation for 5 min at 2000 r/min in an Eppendorf centrifuge, and the supernatant was cleared of cell debris by centrifugation for another 5 min at 14000 r/min

HBV vector-based mouse model

For the *in vivo* experiments, 6 to 8 wk-old female BALB/c mice were used. A total of 18 mice were randomly divided into 3 groups(6 mice in each). Replication competent pHBV1.3 (10 μg) and APOBEC3G expression vectors pXFA3G (10 μg) or pXF3H (10 μg) control plasmid DNA were co-injected into the tail vein of mice in a volume of Ringer's injection equivalent to 9% of the mouse body weight[12,13]; the total volume was delivered within 5 s. The mouse sera were collected at indicated time after hydrodynamic injection, and secreted HBsAg levels and HBV DNA content were measured. All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

HBsAg and HBeAg assays and western blot analysis

Levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera (1:100 diluted) of the treated mice were determined using ELISA (Shanghai Shiye Kehua Company, China). For western blot analysis, cytoplasmic lysates were incubated with 1 volume 2 x loading buffer containing 10% beta-mercaptoethanol for 10 min at 95℃ before loading onto a 12.5% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane *via* electroblotting. The membranes were incubated with HBV core specific rabbit antiserum (Santa Cluz, USA) or with anti-hemagglutinin fusion epitope monoclonal antibody (Santa Cluz, USA) followed by horseradish peroxidase-conjugated mouse anti-rabbit antibody. Proteins were visualized *via* enhanced chemiluminescence (Roche, Germany).

HBV DNA purification and analysis

The method for purification of cytoplasmic core-associated HBV DNA was adapted from Pugh *et al*[14]. Briefly, Huh7 cells were disrupted in lysis buffer (100 mmol/L Tris- HCl pH 8.0, 0.2% NP-40). The cell lysate was clarified by centrifugation at 13000 r/min for 1 min to pellet nuclei and insoluble material. The supernatant was adjusted to 6 mmol/L MgOAc2 and incubated for 2 h at 37℃ with 200 μg/mL of DNase I and 100 μg/mL of RNase A. Following digestion, the lysate was centrifuged for 1 min at 13000 r/min. The supernatant was incubated for 1 h at 55℃ after addition of 10 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl and 200 μg/mL of proteinase K. Finally, the sample was extracted with phenol: chloroform. The DNA was ethanol precipitated, resuspended in TE pH 8 (10 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA) and digested with 100 ng/μL of RNase A for 30 min at 37℃. Purified DNA was subjected to Southern blot analysis. DNA samples were loaded onto 1.3% agarose gels, blotted onto nylon membranes, and probed with a Dig-labeled full-length HBV genome in EasyHyb hybridization solution (Roche, Germany).

For quantitative PCR, 100 μL mouse serum was adjusted to 6 mmol/L MgOAc2 and incubated for 2 h at 37℃ with 200 μg/mL of DNase I and 100 μg/mL of RNase A. Following proteinase K digestion, the sample was extracted with phenol: chloroform. HBV DNA levels were analyzed with the Light Cycle real-time PCR system (Roche, Germany), and primer sequences as follows: 5' -TCACAATACCGCAGAGTC-3' (nt231-248, forward), 5'

- AGCAACAGGAGGGATACA-3' (nt569-552, reverse). The pHBV1.0 vector containing the full-length HBV genome was used as a standard curve to calculate HBV copies per milliliter of serum.

RNA isolation and analysis

For Northern blot analysis, total RNA was extracted from transfected cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Twenty microgram RNA was separated by electrophoresis on a 1% agarose gel and transferred onto nylon membrane, hybridized with a DIG-labeled DNA fragment covering the full-length HBV sequence. The probe was generated with a PCR DIG probe synthesis kit (Roche, Germany). For Northern blot analysis, RNA samples were resolved on MOPS-buffered 1.2% agarose gels containing 1.8% formaldehyde followed by capillary transfer onto nylon membranes and hybridization with a ^{32}P-labeled full-length HBV probe.

For quantitative RT-PCR, approximately 20 mg of liver tissue was obtained from mice for total RNA extraction with RNeasy total RNA kit (QIAGEN, Germany), according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA with oligo(dT)$_{15}$ primer in a total volume of 20 μL. Quantitative RT-PCR was performed with the Light Cycle real-time PCR system (Roche, Germany). The PCR primers are as follows, GAPDH: 5'-GTTGTCTCCTGCGACTTCA-3' (forward), 5'-GGTGGTCCAGGGTTTCTTA-3' (reverse); HBV: 5' -TCACAATACCGCAGAGTC-3' (nt231-248, forward), 5' - AGCAACAGGAGGGATACA-3' (nt569-552, reverse). A standard curve was constructed by the simultaneous amplification of serial dilutions of the expression plasmid encoding HBV used as templates. Target cDNAs were normalized to the endogenous RNA levels of the DAPDH. Quantitative amplification was carried out using the SYBR Green kit (Invitrogen, USA). Gene expression was determined using the relative quantification: $\Delta\Delta C_T$ = ($C_{T\ Target}$ - $C_{T\ GAPDH}$) Test - ($C_{T\ Target}$ - $C_{T\ GAPDH}$) Control. C_T is the fractional cycle number that reaches a fixed threshold, C_{Test} is the test of HBV, and $C_{Control}$ is the reference control (RNA from control group). ΔCT is the difference between gene expression in treated cells and reference control cells. The fold increase was calculated using 2-ΔΔCT[14].

RESULTS

Inhibitory effect of APOBEC3G on HBV DNA replication in cell culture

The mammalian hepatoma cells HuH-7 and HepG2 were co-transfected with replication-competent over-length 1.3 fold HBV and various amounts of a CMV-driven expression vector encoding APOBEC3G. In order to determine the efficiency of transfection, Western blot with polyclonal anti-HA and anti-HBV core antibody was used to analyze the expression of APOBEC3G and HBV core protein in co-transfected Huh7 cells. Three days after transfection, core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates and analyzed with Southern blotting. As shown in Figures 1 and 2, APOBEC3G and HBV core protein were expressed

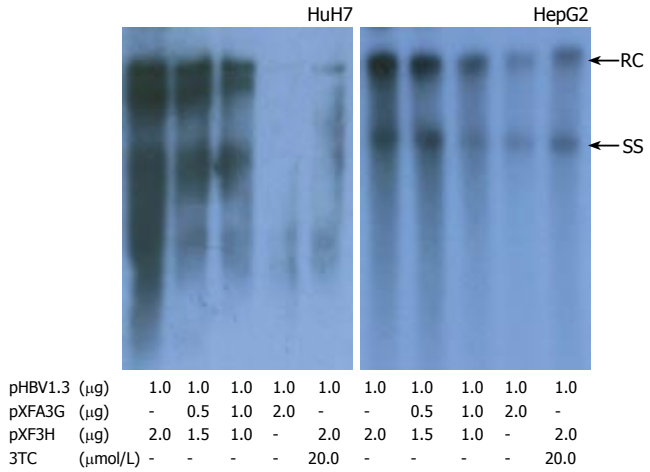

Figure 1 Effect of APOBEC3G on HBV replication in cotransfected hepatoma cells. Cells were treated with lipofectamine 2000 reagents, and viral replicative DNA intermediates were analyzed with southern blotting 3 d after transfection. Numbers at the end of the lines indicate the amount of transfected plasmid DNA in micrograms. HBV, hepatitis B virus; RC, relaxed circular DNA; SS, single -stranded DNA.

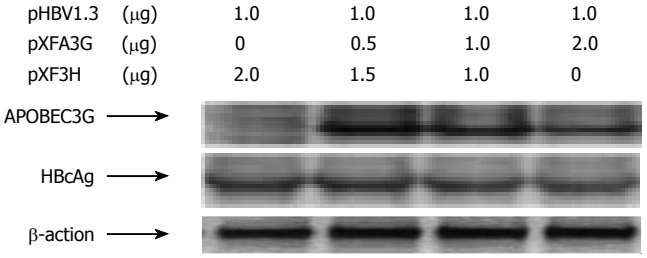

Figure 2 Western blot analysis of cytoplasmic extracts from Huh7 cells cotransfected with indicated plasmids.

in co-transfected Huh7 cells. However, APOBEC3G reduced the level of replicative HBV intermediates in a dose-dependent manner. This inhibitory effect was also observed in HepG2 cell lines.

Inhibition of HBV gene expression by APOBEC3G in cell culture

To evaluate the effects of APOBEC3G on HBV gene expression, HuH7 cells were co-transfected with replication competent over-length HBV(pHBV1.3) and various amounts of CMV-driven expression vector encoding APOBEC3G 3 d after transfection. HBsAg and HBeAg levels were determined by ELISA. As shown in Figure 3, there was also a dose dependent decrease in the levels of extracellular production of HBsAg and HBeAg. However, western blot with polyclonal anti-HBV core antibody indicates that the levels of core protein were unaffected by APOBEC3G (Figure 2). In order to examine the effect of APOBEC3G on HBV RNA levels in co-transfected Huh7 cells, total cellular RNA derived from co-transfected Huh7 cells was hybridized with Dig-labeled probe. Northern blot indicated that the RNA levels of 3.5 kb, 2.4/2.1 kb in co-transfected Huh7 cells were significantly suppressed by APOBEC3G (Figure 4).

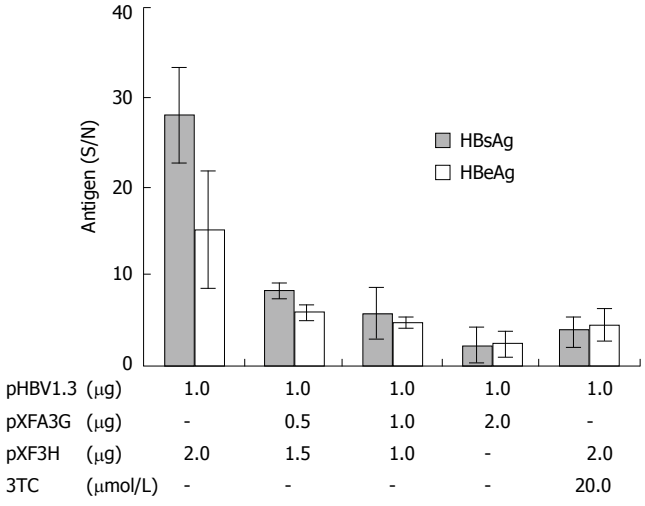

Figure 3 Effect of APOBEC3G on HBsAg and HBeAg secretion in the media of cotransfected Huh7 cells 3 d after transfection. HBsAg and HBeAg levels (S/N) were significantly reduced (error bar indicate standard error). Numbers at the end of the lines indicate the amount of transfected plasmid DNA in micrograms.

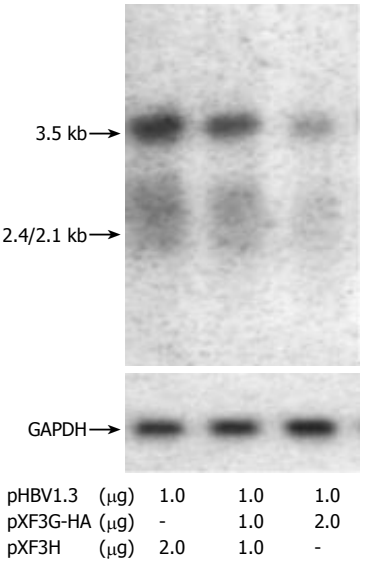

Figure 4 Effect of APOBEC3G on HBV RNA expression. Northern blot analysis of HBV transcripts from Huh7 cells co-transfected with indicated plasmids. The same blot was hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) DNA probe as an internal control.

Inhibition of HBV replication and gene expression by APOBEC3G in mice

To examine whether APOBEC3G could also display HBV inhibition *in vivo*, replication-competent HBV plasmid (pHBV1.3) was co-transfected with pXFA3G or pXF3H(control plasmid) to mouse liver by hydrodynamic injection. As shown previously, hydrodynamic injection of pHBV1.3 into mouse resulted in HBV replication in the liver and secretion of viral antigens to the serum. Consistent with *in vitro* experiments, serum HBsAg and viral DNA in mice which received pHBV1.3 was suppressed dramatically at different time points by APOBEC3G compared with mice without APOBEC3G treatment (Figures 5 and 6). Real time RT- PCR quantification of core-associated HBV RNA in the liver of mice 3 d after injection with APOBEC3G expression plasmid was decreased about 50 times than that of control group (Figure 7).

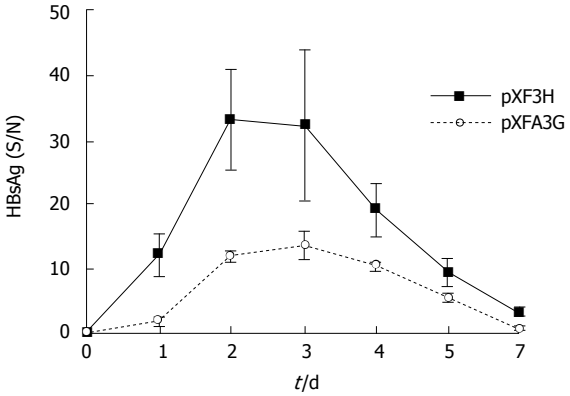

Figure 5 Serum HBsAg levels in APOBEC3G treated mice. HBsAg levels(S/N) in the sera of BALB/c mice were significantly reduced after treatment with the APOBEC3G expression plasmids (error bar indicate standard error) in the indicated time points, *n* = 6 per treatment group.

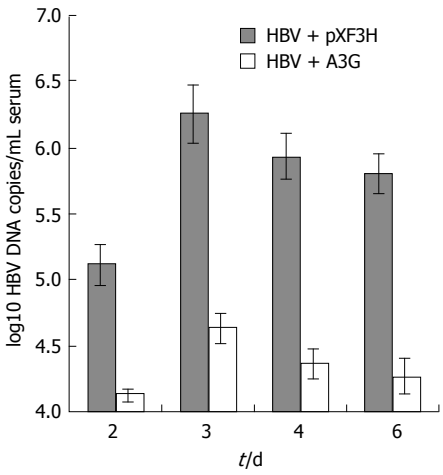

Figure 6 Real time PCR quantification of HBV DNA in the sera of mice treated with APOBEC3G expression plasmid. BALB/c mice were coinjected with pHBV1.3 vector (10 μg) and APOBEC3G expression plasmid (10 μg) 3 d after injection. HBV DNA levels (mean ± SD) of sera were determined by quantitative PCR, *n* = 6 per treatment group.

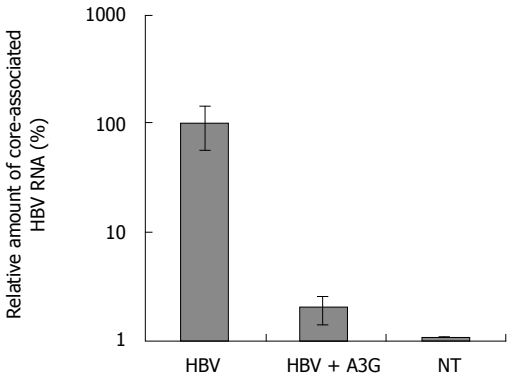

Figure 7 Real time PCR quantification of core-associated HBV RNA in the liver of mice treated with indicated plasmids. BALB/c mice were coinjected with pHBV1.3 vector (10 μg) and APOBEC3G expression plasmid (10 μg) 3 d after injection. The levels of liver HBV RNA were determined by quantitative RT-PCR, normalized to GAPDH mRNA and are reported as a ratio of HBV mRNA/GAPDH mRNA (mean ± SD). *n* = 6 per treatment group.

DISCUSSION

This study explored the effects of human APOBEC3G suppressing HBV DNA production in mammalian hepatoma cells as well as in HBV vector-based mouse model. Our data confirm and extend recent results by

Turelli *et al*[10] who reported A3G-mediated inhibition of HBV DNA production in human HuH-7 hepatoma cells. Previous studies suggested that G-to-A hypermutation can influence HBV pathogenesis. Specific G-to-A changes yield HBeAg-negative HBV variants, often isolated from patients with acute fulminant hepatitis, as well as HBV vaccine escape mutants[17,18]. However, there is no evidence so far that APOBEC-induced lethal hypermutation represents an important innate defense mechanism down regulating hepadnavirus production. APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in transfected HepG2 hepatoma cells[10]. Recently, Rosler *et al*[19] demonstrated that APOBEC3G rendered HBV core protein associated full-length pregenomic RNA nuclease-sensitive. APOBEC3G-mediated editing of nucleic acids does not seem to represent an effective innate defense mechanism for hepadnaviruses.

During the course of an acute infection, HBV DNA clearance apparently occurs through noncytopathic mechanisms in which IFN-γ and TNF-α play an important role[20,21]. APOBEC3G might participate in this type of antiviral response. Although APOBEC3G is not normally expressed in the liver[10,22], recent study shows that APOBEC3G is induced by interferon-α stimulation in human hepatocytes and human primary monocyte-derived macrophages[16,23]. It is possibly induced by interferon in the course of HBV infection. Several studies have shown that IFNs inhibit HBV replication *in vitro* in human hepatoma cells and HBV transgenic mice *in vivo*[24-26]. Currently, IFN-α is approved for treatments of chronic hepatitis B. We speculate that APOBEC3G might be responsible for the anti-HBV action of IFNs in hepatic inflammation. Further analyses will be necessary to determine whether APOBEC3G play roles in the host innate defense against hepatitis viruses *in vivo*.

The hydrodynamic delivery of nucleic acids in the mouse was described by Liu *et al*[11], who showed that the vast majority of the injected nucleic acid was delivered to the liver by this technique. Yang *et al*[27] first demonstrated that hydrodynamic injection of a replication-competent HBV vector resulted in high levels of HBV replication in the livers of injected mice. In the vector-based model, HBV replicates in the liver of immunocompetent mice for 7 to 10 d, resulting in detectable levels of HBV RNA and antigens in the liver and HBV DNA and antigens in the serum. Several reports have documented the use of HBV vector model to examine the *in vivo* activity of co-HDI administered HBV-targeted unmodified siRNAs[28,29] or vector-expressed short hairpin RNAs in silencing HBV gene expression[30]. Most notably, we have demonstrated *in vivo* activity of APOBEC3G expression vector *via* standard intravenous administration. This is the first demonstration of APOBEC3G *in vivo* activity in a hepatitis animal model with a clinically viable route of administration. Although we have seen a more than 1.5log10 reduction of serum HBV levels with APOBEC3G in an HBV mouse model, however, the potential contribution of toxicity from the APOBEC3G has not been ruled out and needs to be investigated further.

Our work demonstrates APOBEC3G effectively inhibits HBV replication in culture cells and mammalian liver, suggesting that such an approach could be useful in the treatment of HBV infection. However, whether suppression of viral replication by APOBEC3G plays a role during natural HBV infection seems speculative at present, because expression of APOBEC3G in human liver tissue has not yet been shown. Nevertheless, a better understanding of the mechanisms of APOBEC3G action may help identify new therapeutic strategies against chronic hepatitis B.

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BASIC RESEARCH

Mechanism for Src activation by the CCK2 receptor: Patho-physiological functions of this receptor in pancreas

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Abstract

AIM: To investigate *in vivo*, whether CCK2 receptors (CCK2R) regulate proteins known to play a crucial role in cell proliferation and cancer development and analyse *in vitro* the molecular mechanisms that lead to Src activation; in particular, to identify the domains within the CCK2R sequence that are implicated in this activation.

METHODS: The expression and activation of Src and ERK were studied *in vivo* using immunofluorescence and western-blot techniques. We used pancreatic tissues derived from wild type or Elas-CCK2 mice that expressed the CCK2R in pancreatic acini, displayed an increased pancreatic growth and developed preneoplastic lesions. The pancreatic tumor cell line AR4-2J expressing the endogenous CCK2R or COS-7 cells transiently transfected with wild type or mutant CCK2R were used as *in vitro* models to study the mechanism of Src activation. Src activation was measured by *in vitro* kinase assays, ERK activation by western blot using anti-phospho-ERK antibodies and the involvement of Src in gastrin-induced cell proliferation by MTT test.

RESULTS: We showed *in vivo* that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, led to the activation of Src and the ERK pathway. Src was activated upstream of the ERK pathway by the CCK2R in pancreatic tumoral cells and contributed to the proliferative effects mediated by this receptor. *In vitro* results demonstrated that activation of the Src/ERK pathway by the CCK2R required the NPXXY motif, located within the CCK2R sequence at the end of the 7th transmembrane domain, and suggested the putative role of Gq in this mechanism.

CONCLUSION: Deregulation of the Src/ERK pathway by the CCK2R might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development.

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Key words: Gastrin; Src; Pancreas; CCK2 receptor

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INTRODUCTION

The CCK2 receptor (CCK2R or CCKBR) is a G protein-coupled receptor, mainly coupled to Gq proteins[1]. Initially implicated in gastrin-mediated acid secretion CCK2R is now recognized to mediate mitogenic and anti-apoptotic effects on gastrointestinal and pancreatic cells[2]. In the transgenic mice, Elas-CCK2, CCK2R expression has been targeted in pancreatic acinar cells using transcriptional elements of the elastase-1 promoter[3]. Using this model, we have recently reported an increase in pancreatic growth as well as an acinar to ductal transdifferentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis that precedes the development of tumours[3]. Similar observations have been reported in two other transgenic models overexpressing TGF-α or a Kras mutant in exocrine pancreas[4,5].

Src family kinases are non receptor protein tyrosine kinases that mediate a wide variety of biological effects including cell survival, adhesion and proliferation. They are activated by many growth factor receptors. In addition, p60-Src is well established as an oncogene, and overexpression of Src tyrosine kinase has been described in human pancreatic adenocarcinomas[6]. Recently, Src inhibition by

AZM475271 demonstrated significant antitumorigenic and anti-metastatic activity in an orthotopic nude mouse model for human pancreatic cancer[7]. However to our knowledge, there is currently little or no information regarding the deregulation of Src kinases in early stages of pancreatic carcinogenesis.

It is well established that numerous G protein-coupled receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. Molecular mechanisms of Src activation by CCK2R as well as the role of this kinase and related signalling pathways in the pathophysiological functions of CCK2R *in vivo* remain largely unknown. This study had two main aims: First, to investigate *in vivo*, using the Elas-CCK2 mouse model, whether CCK2R is involved in the regulation of proteins known to play a crucial role in cell proliferation and cancer development; Second, to analyse *in vitro* the molecular mechanisms that lead to Src activation and in particular, to identify the domains within the CCK2R sequence implicated in this activation.

MATERIALS AND METHODS

Animals

Homozygous Elas-CCK2 mice used in this study have been previously described[3]. At least 3 homozygous Elas-CCK2 mice in a B6SJLF1 background and 3 corresponding control littermate mice were used (six months old). Mice were reared in routine animal facility of the IFR31 and maintained on a 12:12 h light-dark cycle. All the experiments were performed during the daytime. All procedures were approved by the IFR31 animal facility care committee.

Antibodies and materials

GAPDH was provided by Chemicon; phospho-tyr418-Src (IF and WB) by Biosource; ERK, Src (IF and WB) by Santa Cruz Biotechnology; phospho-ERK (IF and WB) by Cell Signaling; SRC (IP) by Oncogene Research Product; PP2, GP2A by Calbiochem.

Immunofluorescence staining

Mice were killed by decapitation and the pancreas was excised, fixed in Bouin's solution and embedded in paraffin using standard techniques. Immunofluorescence staining was performed as previously described[8]. The detection was done using secondary antibodies coupled to Alexa Fluor 488. Slides were analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. For semi-quantitative comparisons, identical volumes of antibody were used for all samples and identical exposure times taken.

Western-blot analysis

Western-blot analyses were performed on dispersed acini from mouse pancreas prepared as previously described[9], and on cell lysates or immunoprecipitates from AR4-2J or COS7 cells stimulated or not with gastrin. Fractions, containing identical levels of proteins, were separated by SDS-PAGE and analyzed by western-blot with the indicated antibodies as described previously[9].

Cell culture and proliferation assay

AR4-2J cells and COS-7 cells were grown in DMEM supplemented with 10% and 5% fetal calf serum (FCS), respectively, at 37℃ in a 95% air, 5 mL/L CO_2 atmosphere. For proliferation assays, an optimal number of AR4-2J cells (4×10^4 cells) were plated in 35-mm dishes, serum-starved for 24 h, then treated for 48 h with gastrin (10 nmol/L). When indicated, cells were incubated with PP2 (10 μmol/L). Cells were counted by using a Coulter electronic counter.

Src kinase assay

After gastrin stimulation, cells were lysed and Src immunoprecipitated with specific antibodies. Kinase assays were performed and analyzed as previously described[10]. Proteins were separated by SDS-PAGE and the gel autoradiographed.

Construction of mutant receptor cDNAs and transient transfection

Mutant CCK2R, N386A-CCK2R was previously described[9]. Plasmids coding for wild type or mutant CCK2R (6 μg) were transiently transfected into COS-7 cells using the DEAE/dextran method as described previously[1].

Statistical analysis

Data were expressed as mean ± SE and Student's *t* test was performed using "GraphPad Prism". $P < 0.05$ was taken as significant.

RESULTS

Src status in Elas-CCK2 mice

Src activation was analyzed by immunohistochemical methods on pancreatic sections from control and Elas-CCK2 mice of 6 month old using antibodies specific for total Src or detecting the activated form of the protein phosphorylated on tyrosine 418 (P-Src). Acinar tissues derived from Elas-CCK2 mice demonstrated higher levels of Src activation as compared to control mice (Figure 1A, upper panels). In contrast, total Src protein expression was unchanged in the two mouse models (Figure 1A, lower panels).

To confirm and quantify Src activation, western-blot analyses were performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice. Src activation in Elas-CCK2 mice was significantly elevated compared to controls (Figure 1B). Thus, the expression of the CCK2R in mouse pancreatic acini induced Src activation.

The ERK pathways in Elas-CCK2 mice

Activation of the ERK pathways by gastrin has previously been described *in vitro*[10,11]. However, there is currently no *in vivo* information about the status of the ERK pathways in gastrin signaling. Immunofluorescence analysis was per-

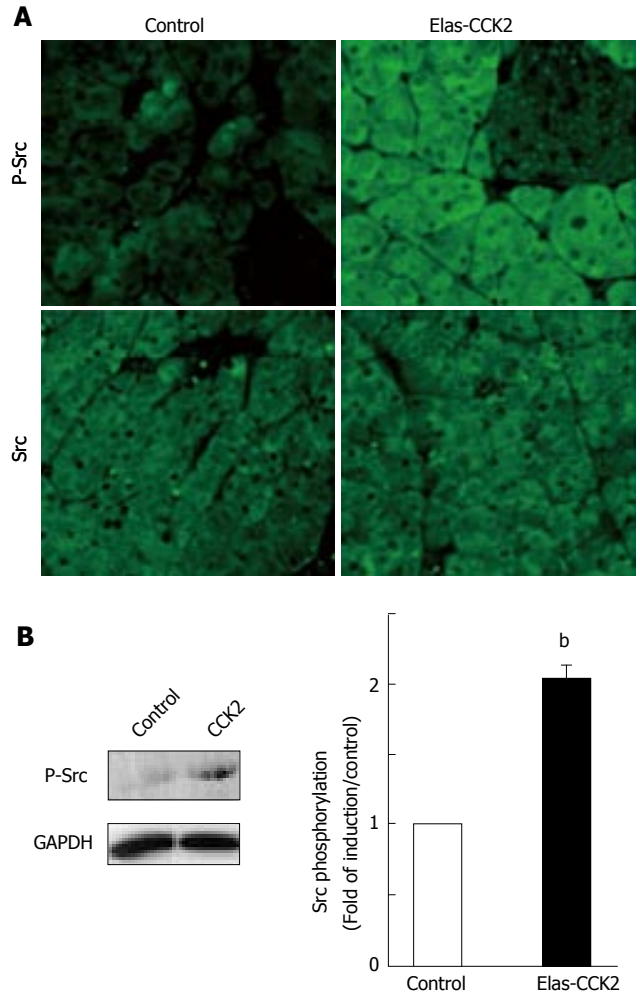

Figure 1 CCK2R expression in acini of Elas-CCK2 mice induced the activation of Src. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or western-blots on lysates from isolated acinar cells were performed using antibodies specific for total Src (SRC) or detecting the activated form of the protein, PY418-Src (P-Src) as indicated. Representative data from 3 experiments (3 different animals in each group) are shown. **A:** Original magnification: 40 X; **B:** Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Results of western-blots quantification are presented as mean ± SE. $^{b}P < 0.01$ *vs* control.

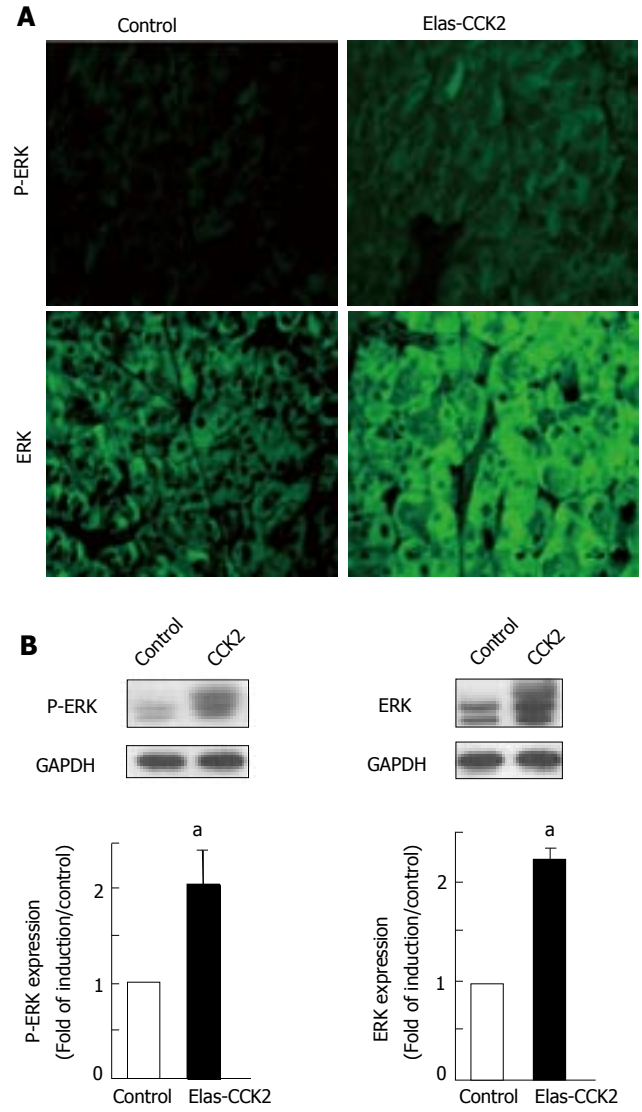

Figure 2 CCK2R expression in acini of Elas-CCK2 mice induces the activation of the ERK pathway. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or western-blots on lysates from isolated acinar cells were performed using antibodies specific for total ERK (ERK) or detecting the activated form of the protein, Phospho-ERK (P-ERK) as indicated. Representative data from 3 experiments (3 different animals in each group) are shown. **A:** Original magnification: 40 X; **B:** Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Results of western-blots quantification are presented as mean ± SE. $^{a}P < 0.05$ *vs* control.

formed using antibodies specific for dually phosphorylated active ERK (P-ERK). Results demonstrated an increased immunoreactivity in acinar tissue of transgenic mice as compared to control mice (Figures 2A, upper panel). In addition, pancreatic acinar tissues of Elas-CCK2 mice also showed a higher level of total ERK expression as compared to control mice (Figure 2A, lower panel). These results were confirmed by western-blots performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice (Figure 2B). Overall, these results were consistent with an upregulation of the ERK pathway in the pancreas of mice expressing the CCK2R.

Src activation by CCK2R in tumour pancreatic acinar cells

The pancreatic tumour cell line AR4-2J, that exhibits an acinar phenotype, was previously shown to express endogenous CCK2R[12]. We used this model to analyse the role of Src in the pathophysiological functions of the CCK2R and the molecular mechanisms potentially involved in Src activation by this G protein-coupled receptor.

We first confirmed that the CCK2R was able to induce Src activation. *In vitro* tyrosine-kinase assays were performed in anti-Src immunoprecipitates from cell lysates containing equal amounts of protein. We detected a rapid and significant increase in Src activation 15 s after stimulation of the CCK2R by gastrin (Figure 3A).

In order to address the role of Src family kinases in proliferation of tumour acinar cells induced by CCK2R, we measured AR4-2J proliferation in the presence or absence of the Src specific inhibitor, PP2, 48 h after gastrin stimulation. CCK2R activation by gastrin induced a significant increase of cell proliferation. Treatment of cells with PP2 totally inhibited CCK2R-induced AR4-2J proliferation (Figure 3B).

We previously reported that CCK2R associates with the αq subunit of heterotrimeric G-proteins[1]. We therefore

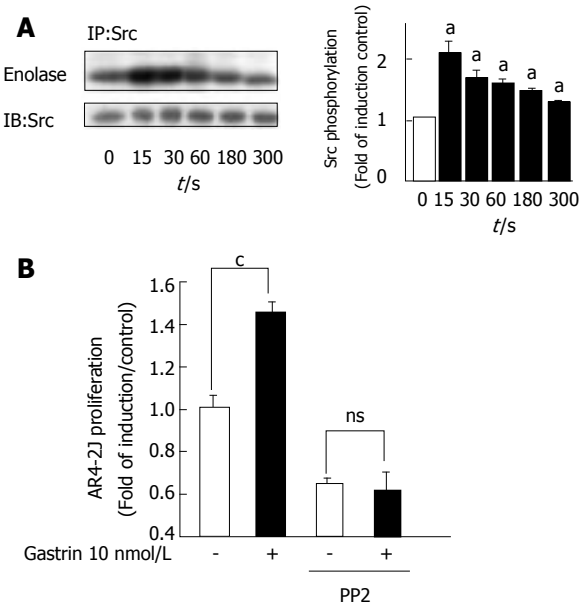

Figure 3 Role of Src in proliferation of tumour pancreatic acinar cells induced by the CCK2R. AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (**A**) or 15 s (**B**). **A:** Src activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. $^{a}P < 0.05$ *vs* control. **B:** Serum-starved AR4-2J cells were treated with Gastrin for 48 h in the presence or absence of PP2 (10 μmol/L), and the proliferation determined as described in Methods. Data are presented as mean ± SE. $^{c}P < 0.001$ *vs* control.

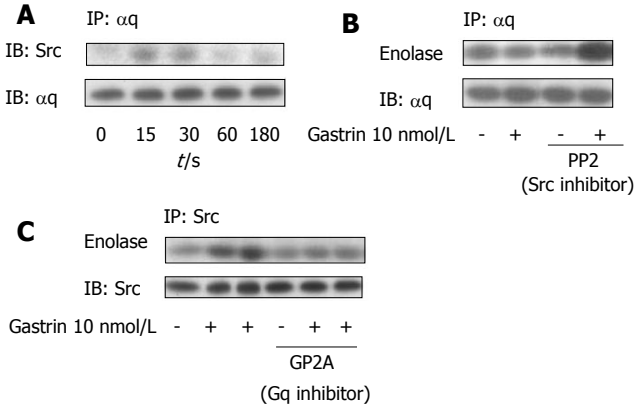

Figure 4 Src activation by the CCK2R in tumour pancreatic acinar cells (**A-C**). AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (**A**) or for 15 s (**B**, **C**). When indicated, cells where pretreated with 10 μmol/L of PP2 or GP2A for 30 min. **A**: Cell lysates were immunoprecipitated (IP) with an anti-αq antibody and immunoblotted (IB) with the anti-Src antibody. The blots were also probed with the antibody used for immunoprecipitation to ensure equal loading of proteins; **B**, **C**: following immunoprecipitation with an anti-αq or an anti-Src antibody, Src activity was determined as described in Methods. Immunoprecipitated proteins were also analyzed by western blot using the anti-αq or anti-Src antibodies as indicated.

tested the hypothesis that Gαq might be involved in gastrin-induced Src activation.

Src proteins were immunoprecipitated with specific antibodies and their association with Gαq was analyzed by western blot with an anti-αq antibody. An increase in the amount of Src proteins coprecipitated with Gαq was detected in response to gastrin (Figure 4A). This effect was time-dependent and the kinetic correlated with that of Src activation in this cellular model. We then

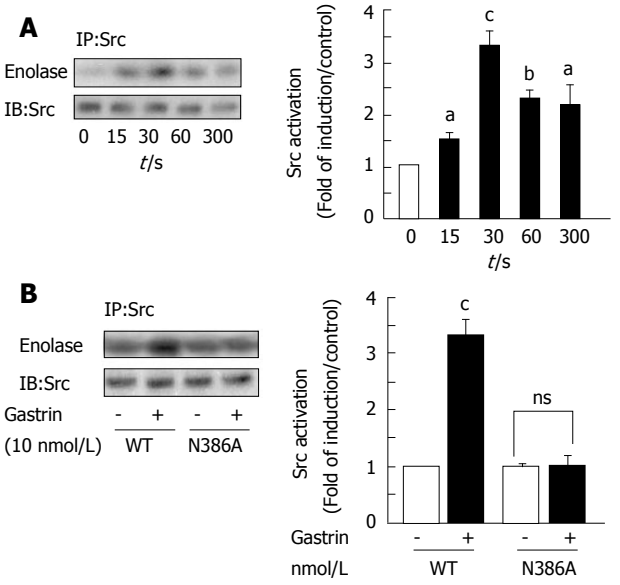

Figure 5 Involvement of the NPXXY motif in Src activation by the CCK2R. **A:** COS-7 cells transfected with the human CCK2R were stimulated with Gastrin (10 nmol/L) for the time indicated. Src kinase activity was determined as described in Methods. Immunoprecipitated proteins were also analyzed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.001$ *vs* control; **B:** COS-7 cells transfected with the WT or N386A mutant CCK2R were stimulated or not with Gastrin (10 nmol/L) for 30 s and Src kinase activity determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. $^{c}P < 0.001$ *vs* control.

examined whether Src-like tyrosine kinase activity was in association with Gαq following gastrin stimulation. An increase in tyrosine kinase activity was detected in anti-Gαq immunoprecipitates after gastrin stimulation which was abolished when samples were treated with the specific Src inhibitor, PP2 (Figure 4B). In addition, pretreatment of the AR42J cells with a specific Gαq inhibitor, GP2A, completely blocked the activation of Src by gastrin (Figure 4C).

Mechanism of CCK2R-induced Src activation in COS-7 cells

To further investigate the molecular mechanisms involved in Src activation by the CCK2R we used COS-7 cells transiently transfected with cDNAs coding for the human wild type CCK2R (WT-CCK2R) or mutant CCK2R. We first validated this model for CCK2R-induced Src activation. Lysates from cells stimulated or not with gastrin were immunoprecipitated with anti-Src antibodies and kinase assays performed as described in methods. A rapid activation of Src (15 s), still detectable at 1 and 5 min, was found in response to Gastrin (Figure 5A). Western-blot analysis for Src protein expression revealed an equal amount of the protein in transfected cells.

Recently, we reported that the NPXXY motif (X represents any amino acid), located at the end of the 7th transmembrane domain of the CCK2R, was involved in Gq-dependent signaling pathways induced by the CCK2R[1,9]. In particular, mutation of the asparagine (N) into alanine (A) inhibits Gq-dependent pathways such as inositol triphosphate (IP3) production. We therefore tested the in-

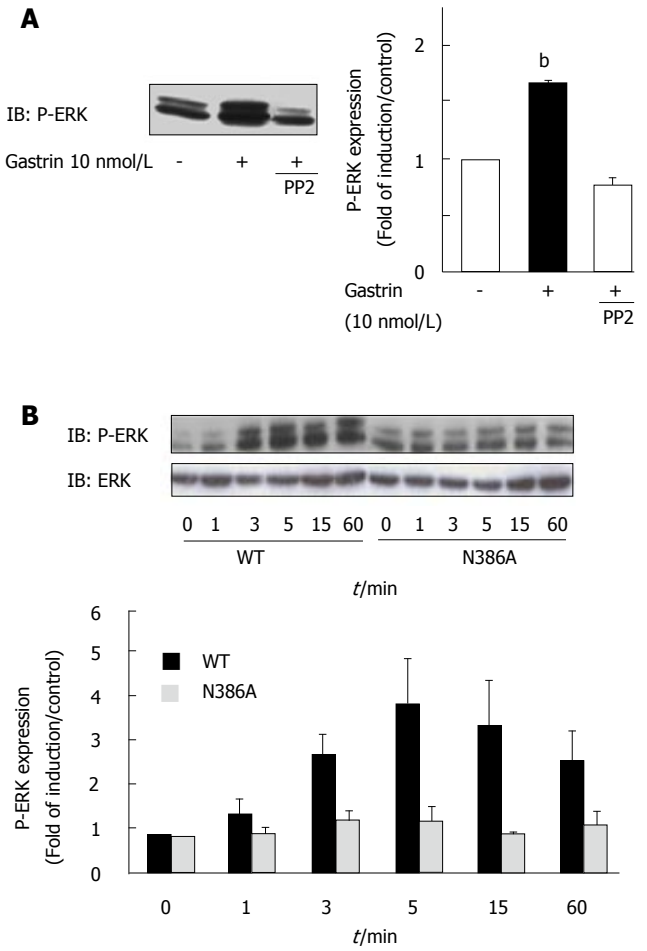

Figure 6 Involvement of the NPXXY motif in ERK activation by the CCK2R. COS-7 cells transfected with the WT or N386A mutant CCK2R were stimulated or not with Gastrin (10 nmol/L) for 5 min (**A**) or the time indicated (**B**). When indicated, cells were pretreated with the Src inhibitor PP2 (10 μmol/L). Equal amounts of protein were analyzed by western-blot using anti-phospho-ERK antibodies. Blots were also reprobed with antibodies directed against total ERK proteins. Results of autoradiography quantification are presented as mean ± SE. $^{b}P < 0.01$ *vs* control.

volvement of the NPXXY motif in Src activation by the CCK2R.

cDNAs coding for the WT receptor or the N386A mutant were transfected in COS-7 cells and Src activation was studied in response to Gastrin. Figure 5B clearly demonstrates that the CCK2R mutant cannot induce Src activation in response to Gastrin in contrast to the WT receptor.

In addition, we demonstrated that ERK activation following gastrin stimulation was completely blocked by the Src inhibitor PP2, indicating that CCK2R-induced ERK activation was totally Src-dependent in this cellular model (Figure 6A). As expected, activation of the ERK pathway was also blocked when the CCK2R was mutated on the NPXXY motif (Figure 6B). Thus, our results demonstrated the involvement of the conserved NPXXY motif within the receptor sequence in the activation of the Src/ERK pathway by the CCK2R, and suggested putative role of Gq in this mechanism.

DISCUSSION

It is well demonstrated that numerous G protein-coupled receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. In the present study our results demonstrate that CCK2R-induced Src activation requires the NPXXY motif, and suggest a putative role of Gq in this mechanism. Indeed, this motif was previously described in Gq-dependent signalling pathways[1,9]. In addition, we showed that Src and Gαq are within one protein complex and we observed a Src-like tyrosine activity associated with Gq. However, despite the appearance of Src and Gαq in the same immunocomplex, we cannot exclude the possibility that Src activation by Gαq requires other protein. Our study also reveals that downstream of Src, activation of the ERK pathway by the CCK2R also requires the NPXXY motif.

The direct interaction of the Src SH3 domain with proline-rich motifs has been reported for the β3 adrenergic and the purinergic P2Y2 receptors[13,14]. Src family kinases also possess an SH2 domain that could directly bind to tyrosine-phosphorylated β2 adrenergic receptor[15]. We tested these two hypotheses in Src activation by the CCK2R. Indeed, the CCK2R has two proline-rich sequences which might interact with SH3 domains. In addition, it also has an ITIM-like motif within the C-terminus tail (LSYTTI) that is potentially phosphorylated on tyrosine residues, in turn leading to the recruitment of PLCγ through its SH2 domain[16]. Mutation of the prolines into serines, as well as the replacement of the tyrosine 438 of the ITIM-like motif by a phenylalanine did not affect the activation of Src by the CCK2R in COS-7 cells (data not shown).

Transactivation of receptor tyrosine kinases is another mechanism by which G protein-coupled receptors activate Src family kinases. Recently, the CCK2R has been shown to transactivate the EGF receptor[17-19]. However, this mechanism seems to be dependent on the cellular model. In the present study, we have observed that inhibition of the EGF receptor by AG1487 did not abolish CCK2R-mediated Src activation in AR42J and COS7 cells (data not shown).

Our study also shows that Src is activated upstream of the ERK pathway by the CCK2R in pancreatic tumor cells and contributes to the proliferative effects mediated by this receptor.

The development of cancer is thought to be dependent on the deregulation of normal signaling pathways involved in cell proliferation, thus conferring a growth advantage to cells. The role of Src and the ERK pathway in the regulation of cell growth is well documented. These signalling molecules have been implicated in the proliferative effects induced by tyrosine kinase receptors, cytokine receptors and G protein-coupled receptors. Activation of Src and ERK proteins has been observed in several human cancers and may contribute to the neoplastic phenotype. However, there is currently very little information regarding the role of the SRC/ERK pathway in the early stages of pancreatic carcinogenesis.

Here we report that the expression of the CCK2R in mouse pancreatic acinar tissue leads to strong activation of the Src tyrosine kinase and the ERK pathway. These transgenic mice display an increased growth of the pancreas

and preneoplastic lesions. They also develop pancreatic tumors with a ductal phenotype similar to what is observed in human pancreatic cancers. Deregulation of the Src/ERK pathway by the CCK2R in these transgenic mice might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development. In addition, while several studies have reported the *in vitro* activation of Src and ERKs by gastrin[10,11,20], this study is the first to demonstrate *in vivo* the up-regulation of the Src/ERK pathway by CCK2R.

In summary, our study describes the mechanism by which the CCK2R, a GPCR mainly coupled to Gq, activates Src. Our results show the involvement of the NPXXY motif within the receptor sequence in this activation, and suggest the putative role of Gq in this mechanism. Moreover, in pancreatic tumoral models we demonstrate *in vitro* and *in vivo* that the CCK2R activates the Src/ERK signaling pathway, a transduction cascade upregulated during tumorigenesis in human.

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BASIC RESEARCH

Effect of rapamycin on hepatic osteodystrophy in rats with portasystemic shunting

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Abstract

AIM: To study if T-cell activation related to portasystemic shunting causes osteoclast-mediated bone loss through RANKL-dependent pathways. We also investigated if T-cell inhibition using rapamycin would protect against bone loss in rats.

METHODS: Portasystemic shunting was performed in male Sprague-Dawley rats and rapamycin 0.1 mg/kg was administered for 15 wk by gavage. Rats received powderized chow and supplemental feeds to prevent the effects of malnutrition on bone composition. Weight gain and growth was restored after surgery in shunted animals. At termination, biochemical parameters of bone turnover and quantitative bone histology were assessed. Markers of T-cell activation, inflammatory cytokine production, and RANKL-dependent pathways were measured. In addition, the roles of IGF-1 and hypogonadism were investigated.

RESULTS: Portasystemic shunting caused low turnover osteoporosis that was RANKL independent. Bone resorbing cytokine levels, including IL-1, IL-6 and TNFα, were not increased in serum and TNFα and RANKL expression were not upregulated in PBMC. Portasystemic shunting increased the circulating CD8+ T-cell population. Rapamycin decreased the circulating CD8+ T-cell population, increased CD8+ CD25+ T-regulatory cell population and improved all parameters of bone turnover.

CONCLUSION: Osteoporosis caused by portasystemic shunting may be partially ameliorated by rapamycin in the rat model of hepatic osteodystrophy.

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Key words: Osteopenia; Liver disease; Portasystemic shunting; T-lymphocyte; Rapamycin

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INTRODUCTION

Metabolic bone disease is a common complication of longstanding liver disease[1,2]. Mechanisms underlying bone loss remain poorly understood and may involve imbalances in bone turnover and mineralization defects. We have shown that portasystemic shunting, a complication of advanced chronic liver disease, is a major pathogenic factor causing bone loss in rats[3].

Excessive osteoclast activity, resulting in localized or generalized bone loss, occurs in various conditions associated with immune activation[4,5]. Activated T-cells express receptor activator of nuclear factor-κB ligand (RANKL) that bind to RANKL receptor on osteoclasts

activating osteoclastogenesis and bone loss[4]. This pathway produces TNFα, IL-1, IL-6, IL-7, and M-CSF all of which have been implicated in bone loss[6-10]. Blockage of RANKL by osteoprotegerin protects against bone loss[4].

The profound impact of activated T-cells on bone has been established. Osteopenia develops in ctla 4-/- mice where T-cells are spontaneously activated, whereas bone loss fails to occur in T-cell deficient nude mice even following ovariectomy[11]. Further, RANKL expressing T-lymphocytes obtained from diseased rheumatoid arthritis joints transform healthy monocytes into osteoclast-like cells[12]. We hypothesized that endotoxin-mediated T-cell activation related to portasystemic shunting may result in osteoclast-mediated bone loss through RANKL dependent pathways.

Employing biochemical parameters of bone turnover and quantitative bone histology, this study aimed to characterize the nature of the bone disease resulting from portasystemic shunting in rats. The role of T-cell activation and inflammatory cytokine production on RANKL-dependent pathways were specifically addressed.

MATERIALS AND METHODS

Animal Experimental design

Ten-week-old male Sprague-Dawley rats weighing 200-300 g were used in all experiments. The rats were housed individually in polypropylene cages at constant room temperature (22℃ ± 2℃), humidity (55%) and 12 h light-darkness cycles. Rats were fed powderized chow (Epol, Johannesburg, South Africa) and water was given *ad libitum*. Rats received a daily 50 mL supplement (Energy 44.52 kJ, protein 0.373 g, carbohydrates 1.456 g, fat 0.373 g). Food intake was measured daily in metabolic cages during four time periods: wk 3, 9, 12 and 15. Ethics committee approval was obtained and animals were treated according to ethical guidelines of the University of Pretoria.

Group Ⅰ: n = 12 PSS. Laparotomy was performed, portal vein was ligated, transected and the distal limb anastomosed end-to-side to the IVC as previously described[3,13].

Group Ⅱ: n = 12 PSS + rapamycin. Portasystemic shunt was performed and rapamycin 0.1 mg/kg administered daily orally by gavage for 15 wk starting 1 wk after surgery. Two rats died during the study period.

Group Ⅲ: n = 12 Sham control. Laparotomy was performed and the portal vein was clamped for 8 min.

Group Ⅳ: n = 12 Sham control + rapamycin. Following laparotomy controls received rapamycin 0.1 mg/kg, orally by gavage starting 1 wk following surgery and continued for 15 wk.

Analytical methods in sera and urine

Blood and urine samples were obtained at baseline and termination and frozen at -70℃. Urine was collected from rats individually housed in metabolic cages. Routine liver tests and testosterone were performed using a Beckman CX-9 and Access auto-analyzers respectively. 25-OH Vit D was determined using scintillation counting detection[14]. Osteocalcin was measured using ELISA kit (Osteometer BioTech, Herlev, Denmark). Cytokine levels were analyzed using an ELISA kit (Biotrac, Amersham, Buckinghamshire, United Kingdom). IGF-1 was measured using an ELISA kit (DRG Inc, Mountainside, USA). Urinary deoxypyridinoline was assessed using an enzyme-labeled immunoassay (Immunolite Pyrilinks-D, Los Angeles, USA).

Liver histology

Rat liver specimens were fixed in 40 g/L buffered formaldehyde and sectioned coronally in 3 μm sections for immunoperoxidase staining utilizing antibodies to ED-1 (1:50 dilution; Serotec, Oxford, UK). Kupffer cells were counted by an experienced histopathologist in ten high power fields (Olympus BX 40, plan 40 x objective) demonstrating the most Kupffer cells. An average per high power field was then calculated.

Bone densitometry

Rats were anaesthetized and bone densitometry was performed using DEXA (DXA QDR 4500^{TM} , Hologic INC, Waltham, USA). Measurement stability was controlled daily by scanning a phantom. Whole-body and high-resolution scans of the right femur were performed at baseline and 16 wk using software for small animals (Hologic, INC, Waltham, USA).

Histomorphometry

Rats received intramuscular injections of 25 mg/kg tetracycline 13 and 3 d prior to termination. At termination the left tibia was removed, stored in 70% ethanol at 4℃, fixed in modified Millonig solution for 24 h, embedded in methyl methacrylate, sectioned at 5 μm and stained by modified Masson technique. Histomorphometric analyses were performed manually using a Merz-Schenk integrating eyepiece. Trabecular bone was analyzed excluding sections within two fields at × 250 magnification from either the growth plate or the cortices. At least 120 fields per animal were counted. Double tetracycline-labeling was assessed on 10 μm thick unstained sections cut from the proximal tibia by fluorescent microscopy using a Merz-Schenk eyepiece. Variables and units used are approved by the American Society for Bone and Mineral Research[15].

Flow cytometry

Peripheral blood obtained at termination were incubated with the following combinations of monoclonal antibodies: fluorescein isothiocyanate/phycoerythrin labeled CD4+/CD25+; CD8+/CD25+ (Immunotech, Beckman Coulter, Inc. Fullerton, USA). Analysis was performed on a Coulter Epics flow cytometer. Lymphocytes were gated on forward and side scatter. The percentage of CD3+ T-cells in the gate was deduced from the percentages for CD4+ and CD8+ T-cells.

Quantitative RT-PCR

Total RNA was extracted from PBMC using the Qiagen RNeasy kit. Aliquots of 8.5 μL were used in a RT-PCR reaction in a total volume of 20 μL (Roche, First strand cDNA synthesis kit, Mannheim, Germany).

Complementary DNA PCR primers were designed from sequences from Genbank or TIGR (Inqaba Biotech, Pretoria, South Africa). *tnfα* (Sense: 5'-atggcccagaccctcac-3', Antisense: 5'-agcacatagacggggcag-3'); *rankl* (Sense: 5'-tggaggatttttcaagctccgg-3'; Antisense: 5'-gccccaaagtacgtcgca-3') and *Gapdh* (Sense: 5'-ggcccctctggaaagct-3'; Antisense 5'-aggtggaggaatgggagt-3'). The reaction mixture consisted of cDNA (1 μL), 10 pmol of each primer, 1 μL LightCycler FastStart DNA Master SYBR Green 1 Mix (Roche, Mannheim, Germany), 500 ng BSA (Gibco, BRL, Gaithersburg, MD) and 3 mol/L $MgCl_2$ in a total volume of 10 μL. FastStart polymerase was activated and cDNA denatured by a pre-incubation of 10 min at 95℃. The template was amplified for 40 cycles of denaturation at 95℃ for 0 s, annealing at 60℃ for 8 s and extension at 72℃ for 12 s. Standard curves were generated from series diluted cDNAs and analyzed using the Light Cycler quantification software.

Analytical methods of bone calcium content

Right femurs were dried for 6 h at 60℃, then ashed for 8 h in a muffle furnace at 600℃. Bones were weighed, and the length and mid-shaft thickness measured. The femurs were dissolved in 3 mL of 6molL HCl and diluted 3000x with demineralized/de-ionized water. Calcium content was determined against a 4 point standard curve (r^2 = 0.9999), using a Perkin-Elmer 3030 atomic absorption spectrophotometer as previously described by our group[3].

Determination of 4-hydroxyproline in left femurs

Dried femurs were dissolved in 10 mL 6 mol/L HCl, at 100℃ for 24 h and centrifuged for 5 min at 4000 r/min at 4℃. n-Tetracosane ($C_{24}H_{50}$) in chloroform was added to the eluates as internal standard. The eluates were dried in nitrogen, the amino acids derivatized with N-methyl-N (*t*-butyldimethylsilyl) trifluoroacetamide and analyzed using gas chromatography as previously reported by our group[16].

Statistical analysis

Data were analyzed using SigmaStat and SigmaPlot for Windows version 4.0. Data were checked for normality and equal variance; if passed, ANOVA was performed and where failed, analysis of variance on ranks (Kruskal-Wallis) was conducted. Results are presented as means ± standard deviation. Analysis of covariance was performed to compare groups with respect to change in BMD from wk 1 to 16. Pair-wise comparisons were performed using Fisher's LSD and for between group comparison Wilcoxon rank sum test was performed. Results were considered significant at $P < 0.05$.

RESULTS

Body mass and food intake

Body mass decreased after surgery in the portasystemic shunted animals despite feeding with powderized and supplemental feeds. Weight gain was restored by wk 3 in the shunted animals and remained parallel to the control groups throughout the study (Figure 1), confirming growth in shunted animals. The rate of weight gain from wk 2 as reflected in the Pearson correlation coefficient did not differ between the shunted and control animals (Figure 1). Powderized food intake remained lower in shunted animals during the first 12 wk of the study ($P < 0.01$). The supplemental feed was consumed by all animals daily. Food intake, corrected for body mass, showed no differences between the groups with total food intake (powderized + supplemental) being higher than the recommended intake for adult laboratory rats (15 g/d). Calcium and Vit D was further supplemented in the powderized chow. By wk 15 no difference in food consumption (powderized feed) was seen (Figure 2). Body mass was still significantly lower in shunted animals at termination (Figure 1).

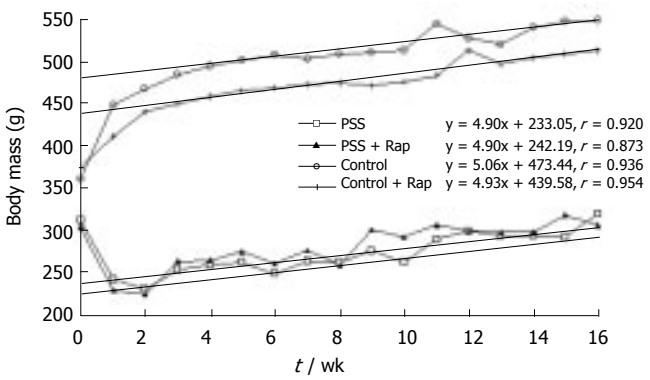

Figure 1 Body mass change per week. *r* = Pearson correlation coefficient.

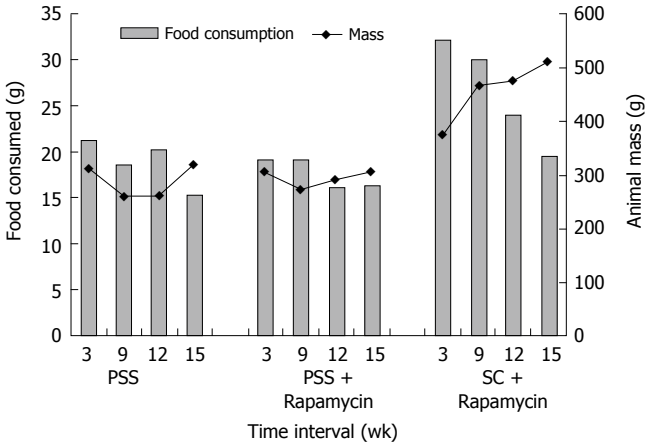

Figure 2 Food intake and body weight in PSS, PSS + rapamycin and SC + Rapamycin.

Serum and urine biochemistries

No differences were seen at baseline. Transaminase levels were elevated in shunted groups and the control group receiving rapamycin. Serum total ALP was elevated in both shunted groups and confirmed to be of hepatic origin by electrophoresis. Serum albumin, testosterone and IGF-1 levels were significantly lower in both shunted groups. 25-OH vit D levels were not different between the groups at 16 wk (Table 1). Serum osteocalcin was significantly lower in the PSS group but not different in the PSS group receiving rapamycin compared to controls (Table 1). A trend towards higher u-DPD levels was observed in

Table 1 Serum and urine biochemistries at 16 wk

	ALP (nkat)	AST (nkat)	ALT (nkat)	Albumin (g/L)	Testosterone ng/L	IGF-I μg/L	25-OH vit D ng/L	Osteocalcin ng/L	u-deoxypyridinoline (μmol/mol creatinine)
Control	2161 ± 369	1182 ± 277	940 ± 187	16.75 ± 0.71	5.41 ± 2.34	879.37 ± 245.51	25.63 ± 4.04	142.91 ± 32.9	48.8 ± 7.9
Control + rapamycin	2762 ± 389[a]	2022 ± 1152[a]	1155 ± 122[a]	15.78 ± 1.3	10.62 ± 9.1	616.02 ± 189.66	29.15 ± 8.00	147.43 ± 34.0	67.12 ± 13.6
PSS	7144 ± 2121[b]	2210 ± 741[a]	1538 ± 243[b]	15.25 ± 0.75[a]	3.55 ± 0.41[a]	290.28 ± 170.90[a]	23.89 ± 5.97	105.57 ± 38.3[a]	61.99 ± 18.7
PSS + rapamycin	8481 ± 3380[b]	3217 ± 1560[a]	1752 ± 549[a]	14.33 ± 1.22[a]	3.45 ± 0.74[b]	241.66 ± 80.58[a]	22.15 ± 5.07	119.61 ± 43.5	93.79 ± 30.6[a]

[a]$P < 0.05$ *vs* Controls; [b]$P < 0.001$ *vs* Controls.

Table 2 Bone analysis at 16 wk

	Whole body BMD mg/cm^2	High resolution BMD mg/cm^2	Femur length (mm)	Mid-shaft thickness (mm)	Femur mass (g)/kg body mass	Ca^{2+} mg/g bone	Hyp/g bone
Control (n = 12)	211.7 ± 9	379.7 ± 46	42.8 ± 1.1	4.7 ± 0.3	1.49 ± 0.1	260 ± 7	3576 ± 1980
Control + rapamycin (n = 12)	210.0 ± 8	377.2 ± 43	42.8 ± 1.3	4.7 ± 0.3	1.53 ± 0.1	258 ± 9	6104 ± 1600[a]
PSS (n = 11)	201.9 ± 8[a]	309.3 ± 63[a]	36.6 ± 0.6[b]	4.1 ± 0.3[a]	1.80 ± 0.2[a]	289 ± 5[b]	2038 ± 878
PSS + Rapamycin (n = 10)	210.5 ± 17	334. ± 64	37.3 ± 1[b]	3.8 ± 0.3[b]	1.81 ± 1.37[b]	297 ± 9[b]	5489 ± 1250[a]

[a]$P < 0.05$ *vs* Controls; [b]$P < 0.001$ *vs* Controls.

Table 3 Quantitative histomorphometry

	Osteoid volume, OV/BV (%)	Relative osteoid volume, OV/TV (%)	Osteoid surface, OS/BS (%)	Osteoid thickness, O.Th (μm)	Osteoblast appositional rate, OAR (μm/d)	Mineralization lag time, MLT (d)	Eroded surfaces, ES/BS (%)	Osteoclastic surfaces, OcS/BS (%)
Control	1.26 ± 0.24	0.15 ± 0.04	8.94 ± 1.27	5.67 ± 0.65	1.03 ± 0.09	5.61 ± 1.08	5.63 ± 0.55	1.07 ± 0.20
Control + Rapamycin	1.34 ± 0.38	0.19 ± 0.06	10.44 ± 1.86	5.30 ± 0.93	1.05 ± 0.08	5.06 ± 1.25	6.35 ± 0.81	1.42 ± 0.24
PSS	3.69 ± 1.43	0.66 ± 0.32	17.48 ± 4.96	8.00 ± 1.32	0.85 ± 0.86	19.7 ± 5.07[a]	3.62 ± 0.63[a]	0.97 ± 0.28
PSS + Rapamycin	1.33 ± 0.19	0.15 ± 0.02	10.05 ± 1.53	5.72 ± 0.86	0.95 ± 0.11	11.02 ± 1.99	4.40 ± 0.62	0.94 ± 0.19

[a]$P < 0.05$ *vs* Controls.

shunted animals compared to control animals. Significantly higher u-DPD levels were seen in control animals receiving rapamycin.

DEXA, bone mass and composition

Mean whole body and femoral BMD were comparable at baseline. At 16 wk whole body and high resolution femoral BMD were significantly lower in portasystemic animals compared to controls (Table 2). BMD in PSS receiving rapamycin did not differ from controls. The change in BMD from baseline to wk 16 using pair-wise comparisons showed that the increase in BMD in the shunted group (group I) was significantly lower compared to the control group ($P = 0.006$). The increase in BMD in the PSS receiving rapamycin was comparable to controls ($P = 0.07$). Shunted animals had lower body mass, femoral length and thickness (Table 2). Femur mass, expressed as a function of body mass, was increased in PSS. Femoral calcium content, expressed as a function of femoral mass (mg Ca^{2+}/mg femur), was not decreased in shunted animals, excluding significant osteomalacia. Femoral hydroxyproline levels tended to be lower in the PSS group compared with controls (Table 2). Unexpectedly, shunted and control rats receiving rapamycin had significantly higher femoral hydroxyproline.

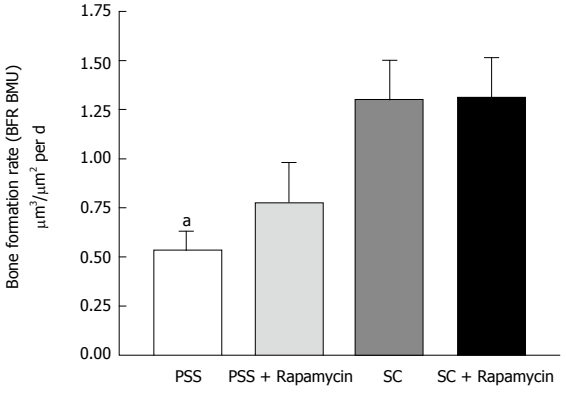

Figure 3 Effect of portasystemic shunting and rapamycin administration on dynamic bone formation in the proximal tibias of rats. BFRBMU = Bone formation rate at the level of the basic multi-cellular unit. (mean ± SE. [a]$P < 0.05$ *vs* SC).

Quantitative Histomorphometry

Osteoid volumes and surfaces, and mean osteoid seam thickness, was higher in the PSS compared with control animals (Table 3). Bone formation was decreased in PSS rats (Figure 3). Mineralization of newly formed osteoid,

Table 4 Cytokine levels, T-lymphocyte subsets and Kupffer cell populations at 16 wk

	IL-1 (ng/L)	IL-6 (ng/L)	TNFα (ng/L)	%CD3+	%CD4+	%CD8+	Ratio CD4+: CD8+	%CD4+ expressing CD25	%CD8+ expressing CD25	Kupffer cells /10 high power fields
Control	22.28 ± 4.39	0	13.6 ± 2.73	74.05 ± 5.49	43.15 ± 3.93	30.9 ± 7.05	1.4	7.96 ± 2.99[d]	10.62 ± 2.64[f]	46.26 ± 7.95
Control + rapamycin	25.86 ± 8.71	0	14.84 ± 2.35	73.34 ± 7.73	47.86 ± 8.31	25.48 ± 6.4	1.8	15.54 ± 9.5[d]	17.9 ± 13.87[f]	40.3 ± 8.46
PSS	22.23 ± 5.12	0.025 ± 0.045	26.15 ± 45.97	93.4 ± 7.97[a]	58.96 ± 9.02	34.47 ± 6.59[b]	1.6	5.38 ± 2.47[c]	6.05 ± 1.99[e]	43.45 ± 8.13
PSS + Rapamycin	26.12 ± 6.21	0.011 ± 0.033	15.5 ± 11.83	73.19 ± 5.66	56.78 ± 5.60	18.39 ± 5.73[b]	3.1	11.38 ± 6.96[c]	19.10 ± 14.58[e]	44.95 ± 9.16

%CD3 + T cells PSS *vs* controls [b]$P < 0.001$ *vs* Controls; %CD8+ T cells PSS *vs* PSS + Rapamycin [d]$P < 0.001$; %CD4 + CD25 + T cells PSS *vs* PSS + Rapamycin [f]$P < 0.001$; %CD4 + CD25 + T cells Control *vs* Control + Rapamycin [h]$P < 0.001$; %CD8 + CD25 + T cells PSS *vs* PSS + Rapamycin [i]$P < 0.001$; %CD8 + CD25 + T cells Control *vs* Control + Rapamycin [k]$P < 0.001$.

reflected in the mineralization lag time, was significantly delayed in PSS rats, accounting for the accumulation of osteoid in these animals (Table 3). Bone resorption over 16 wk, as reflected in the total eroded surfaces, was significantly decreased in PSS animals, although active osteoclastic resorption at the time of sacrifice was comparable among groups (Table 3). Bone turnover in shunted animals was increased by rapamycin as evidenced by increase bone formation and resorption. Osteoid accumulation and the mineralization defect induced by shunting were largely normalized by rapamycin (Table 3).

T-cells, cytokine levels and tnfα and rankl expression

PSS increased the percentage of circulating CD3+ lymphocytes by 1.3 fold ($P < 0.001$) above that for control rats (Table 4). Rapamycin treatment of PSS rats returned the CD3+ T-cell population to that of control rats with an altered ratio of CD4+ to CD8+ T-cells of 3:1. No alteration occurred in either the size of the CD3+ population or the normal ratio of CD4+ to CD8+ T-cells in rapamycin treated control rats (Table 4). PSS did not affect the normal CD4+ or CD8+ T-cells fractions expressing CD25. There was a significant increase ($P < 0.001$) in the percentage of CD4+ and CD8+ T-cells expressing CD25 with rapamycin treatment in both PSS and control rats (Table 4). Serum levels of IL-1, IL-6 and TNFα were similar in all groups (Table 4). RT-PCR showed that TNFα gene expression in shunted and control animals was comparable. RANKL-expression was significantly lower in PSS animals.

DISCUSSION

We previously investigated the contribution of parenchymal inflammation, portal hypertension and portasystemic shunting to the development of metabolic bone disease using rat models. Only portasystemic shunting caused significant bone loss[3]. The present study aimed to delineate the immune responses associated with portasystemic shunting and how this may influence bone loss in the rat. Further, the profound effect of activated T-cells on bone turnover has been well established [11,12]. We set out to study the effect of T-cell inhibition using rapamycin, not shown to have adverse effects on bone composition (in contrast to cyclosporin and tacrolimus), on biochemical and histological bone parameters in rats following portasystemic shunting.

The present study confirmed the adverse effect of portasystemic shunting on skeletal integrity as evident by a significant decrease in whole-body and femoral BMD. Quantitative bone histology documented hyperosteoidosis and an increased mineralization lag time in shunted animals, suggesting a degree of impaired mineralization. Histological evidence of frank osteomalacia was absent. Serum 25-OH vitamin D levels and femoral calcium content were comparable between shunted and control animals, and ALP iso-enzymes in shunted animals originated from liver not bone, supporting the histological evidence that the low BMD observed in shunted animals was mainly the result of osteoporosis.

Femur mass per 100 g body mass was higher in shunted rats compared to controls. Trabecular bone loss is known to occur earlier and more extensive compared to cortical bone loss due to the large surface area available for resorption. The apparent discrepancy between the rise in femur mass per 100 g of body mass in portasystemic shunted animals may be explained by lean body mass being lost earlier and more rapidly than cortical bone mass so that a lag in cortical bone loss gives rise to an apparent increase in bone mass relative to body mass. Low-turnover osteoporosis may occur due to protein-energy malnutrition[17]. IGF-1 and testosterone levels were decreased in the shunted rats. Malnutrition[18] and liver disease[19] have been associated with decreased circulating levels of IGF-1. Low testosterone levels have been previously documented in portasystemic shunted rats[20] with osteoporosis[3]. IGF-1 has previously been implicated in bone loss in chronic liver disease[21,22].

It is not possible to exclude that malnutrition contributed to bone loss in shunted rats. Malnutrition is associated with advanced liver disease although muscle wasting independent of malnutrition may be a feature of liver cirrhosis[23]. Reduced spontaneous locomotor activity due to altered histaminergic neurotransmission can reduce food intake in shunted rats[24]. We could, however, document that food intake in the shunted animals met the nutritional requirements calculated for their body weights during four time periods. However, despite restoring growth and weight gain (Figure 1), osteoporosis still developed in the portasystemic shunted rats. This improved feeding protocol may explain the differences observed in the present study compared to

our previous study[3] In our previous study weight loss was observed throughout the study period and TNFα levels were increased and vitamin D levels decreased in shunted animals. In the current study TNFα and vitamin D levels did not differ from the control animals suggesting a stable model due to improved feeding.

An important observation in the study was that portasystemic shunted animals receiving rapamycin had increased bone formation, whole body and high resolution BMD, as well as increased osteocalcin levels in serum and hydroxyproline content in femurs, compared to shunted animals not receiving rapamycin. These positive changes on bone turnover variables occurred despite lower body mass, IGF-1 and testosterone levels compared to controls. This finding further suggests that malnutrition and decreased IGF-1 and low testosterone levels can only partially explain osteoporosis observed in shunting.

Pro-inflammatory cytokines like IL-1, IL-6, TNFα and RANKL are known to activate osteoclastogenesis and cause high-turnover osteoporosis[9-11,25]. Serum levels of IL-1, IL-6 and TNFα, and *tnf*-α and *rankl* gene expression in PBMC, were not increased in shunted rats. Instead, osteocalcin, a marker of bone formation, was significantly decreased and quantitative histomorphometry showed impaired bone formation, suggesting low-turnover bone disease in shunted rats. Collectively, these observations suggest that the pro-inflammatory cytokines studied, are not responsible for bone loss in portasystemic shunting.

Rapamycin decreased the circulating CD8+ T-cell population in PSS rats. Lymphocyte proliferation inhibition by rapamycin is known to be dependent on the activation pathway of the proliferative signal with CD8+ T-cell proliferation being more affected than CD4+ T-cell proliferation[26-28]. The effect of rapamycin on the CD8+ T-cell population in PSS rats indicate that these cells are more activated than CD4+ T-cells.

Expression of the CD25 marker in this study does not appear to reflect cellular activation. There was no difference between the control group and PSS group with respect to fraction of CD4+ or CD8+ T-cells expressing the CD25 marker, suggesting that the cells expressing CD25 belong to a population of regulatory T-cells (Treg cells). Treg cells in mice and humans are associated with a restriction of most immune responses[29]. No documentation of such cell populations in rats has been made. Rapamycin increased the percentage of CD4+ and CD8+ T-cells expressing CD25 for both normal and shunted rats. This has been proposed to be a component of the immunosuppressive properties of rapamycin[30]. Although not much is known of the function of the CD8+CD25+ T-cell population in rodents, this population in humans has similar immunoregulatory characteristics to the CD4+CD25+ T-cell population[31,32]. Murine studies indicate an inability of CD8+CD25+ T-cells to induce osteoclast differentiation[33]. In shunted rats where the fraction of these cells was increased by rapamycin, increased bone formation was observed. An increase in this population of CD8+ T-cells by rapamycin in normal rats also increased hydroxyproline levels in femurs.

The overall changes in the lymphocyte population seen with rapamycin treatment of shunted rats are associated with an increase in bone formation, whole body and high resolution BMD as well as higher circulating levels of osteocalcin and femoral hydroxyproline content. Although IL-1, IL-6, TNFα and RANKL do not appear to be involved in portasystemic shunting associated osteopenia, other cytokines that are modulated by rapamycin may be implicated. IFNγ suppresses osteoclast activity while macrophage production of IL-12, a major stimulator of lymphocyte IFNγ production is upregulated by rapamycin. T-cell production of IFNγ via an IL-12 mechanism may be important in the bone protection observed in rapamycin treated animals[34,35]. An alternative explanation whereby rapamycin may increase bone formation is through mTOR a member of the phosphoinositide 3-kinase related kinase (PIKK) family, which plays a critical role in transducing proliferative signals mediated through the PI3K/Akt signalling pathway[36]. The mTOR gene is expressed in osteoblasts[37,38] and rapamycin has been shown to up-regulate growth factors like BMP-4 and latent TGF-β binding protein in certain cancer cell lines[39]. This may present an additional pathway by which bone formation is stimulated by rapamycin.

In conclusion, we hypothesized that portasystemic shunting would result in T-cell activation, cytokine mediated osteoclastogenesis and high-turnover osteoporosis. Instead, we documented low-turnover osteoporosis, which was partially ameliorated by rapamycin. A better understanding of the direct mechanisms of rapamycin on bone is required.

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BASIC RESEARCH

Pancreatic regenerating protein (reg I) and reg I receptor mRNA are upregulated in rat pancreas after induction of acute pancreatitis

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Abstract

AIM: Pancreatic regenerating protein (reg I) stimulates pancreatic regeneration after pancreatectomy and is mitogenic to ductal and β-cells. This suggests that reg I and its receptor may play a role in recovery after pancreatic injury. We hypothesized that reg I and its receptor are induced in acute pancreatitis.

METHODS: Acute pancreatitis was induced in male Wistar rats by retrograde injection of 3% sodium taurocholate into the pancreatic duct. Pancreata and serum were collected 12, 24, and 36 hours after injection and from normal controls (4 rats/group). Reg I receptor mRNA, serum reg I protein, and tissue reg I protein levels were determined by Northern analysis, enzyme-linked immunosorbent assay (ELISA), and Western analysis, respectively. Immunohistochemistry was used to localize changes in reg I and its receptor.

RESULTS: Serum amylase levels and histology confirmed necrotizing pancreatitis in taurocholate treated rats. There was no statistically significant change in serum reg I concentrations from controls. However, Western blot demonstrated increased tissue levels of reg I at 24 and 36 h. This increase was localized primarily to the acinar cells and the ductal cells by immunohistochemistry. Northern blot demonstrated a significant increase in reg I receptor mRNA expression with pancreatitis. Immunohistochemistry localized this increase to the ductal cells, islets, and acinar cells.

CONCLUSION: Acute pancreatitis results in increased tissue reg I protein levels localized to the acinar and ductal cells, and a parallel threefold induction of reg I receptor in the ductal cells, islets, and acinar cells. These changes suggest that induction of reg I and its receptor may be important for recovery from acute pancreatitis.

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Key words: Acute pancreatitis; Reg, reg receptor; Taurocholate; Regeneration

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INTRODUCTION

Since its isolation in 1988 by Terazono *et al*[1], much work has been done to elucidate the function of the regeneration protein, reg I. Reg I protein appears to function as a growth factor in the pancreas, as evidenced by its structural similarity to calcium-dependant lectins[2], as well as its mitogenic effect on beta and ductal pancreatic cell lines[3] and its ability to reverse surgically induced diabetes[4,5]. Reg I gene expression has also been found in other tissues including gastric cells[6], and rats with water immersion stress induced gastric lesions demonstrate an increase in reg I expression in gastric enterochromaffin-like cells during healing[7]. Reg I is therefore involved in the regeneration and growth of gastrointestinal tissue. Furthermore, acute pancreatitis induces reg I gene expression and protein production in the pancreas[8,9]. Recently, Kobayashi *et al* isolated the gene for reg I receptor[10], which is part of the exostoses family of genes. Transfection of the gene into the pancreatic β-cell line RINm5F cells resulted in increased mitogenesis after exposure to reg I protein.

Since reg I and its receptor are linked to cellular

mitogenesis and may affect repair of damaged pancreas, we examined the role of their expression in acute pancreatitis.

MATERIALS AND METHODS

Induction of acute necrotizing pancreatitis

Acute necrotizing pancreatitis was induced in male Wistar rats using a 30 g/L sodium taurocholate solution according to a protocol described by Aho *et al* with slight modification[11]. The animals (4 rats/group) were anesthetized with a 1:1 mixture of ketamine (100 mg/mL) and xylazine (20 g/L) dosed at 0.1 mL per 100 g of body mass injected subcutaneously. The abdominal cavity was then entered through a midline incision. The duodenum and pancreas were identified, the common biliopancreatic duct ligated at the hilum of the liver, and a duodenostomy made approximately 1 cm distal to the opening of the biliopancreatic duct into the duodenum. A polyethylene catheter with an inner diameter of 0.58 mm and an outer diameter of 0.965 mm was used to cannulate the pancreatic duct through the duodenostomy. A 30 g/L sodium taurocholate solution was slowly injected at a dose of 1 mL/kg at a constant infusion rate of 0.5 mL/min. The catheter was left in place for 30 min and then removed. The midline incision was then closed in a single layer closure. Control animals consisted of sham operated rats (open laparotomy with immediate closure) and healthy rats.

Harvesting of pancreas and serum

A total of 12 rats were operated on for induction of pancreatitis. Four rats were sacrificed at each time point (12, 24, and 36 h). Four rats per group (unoperated and sham operated) were used for controls. The pancreas was harvested and blood collected by cardiac puncture from each rat. A small portion of the head of the pancreas was fixed in formalin and sent for staining with hematoxylin and eosin (HE) and immunohistochemical staining with reg I mAb[12]. The remaining pancreas was divided in half. One portion was immediately snap-frozen in liquid nitrogen and used for RNA isolation (below), and the other for protein isolation. This was performed by homogenization in buffer containing 125 mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 5 mmol/L piperizine ethane sulfuric acid-Tris (PIPES-Tris) (pH 6.7). The homogenate was centrifuged at 4100 g for 30 min and the supernatant collected and frozen at -20℃. The concentration of total protein in the pancreas homogenates was quantified using the BioRad Protein Assay solution according to the manufacturer's protocol. The blood was centrifuged at 1800 g for 15 min. The supernatant (serum) was collected and stored at -20℃.

Serum amylase activity was determined by enzymatic reaction. Formaldehyde-fixed pancreatic specimens from each rat were stained with HE.

Preparation of reg I cDNA probe

Reg I cDNA probe was prepared by PCR using a plasmid with a reg I insert as a template[3]. The sequences of the oligonucleotide primers are 5'ACGCGTCGACTCATGACTCGCAACAAATATTTC3' and 5'GGCACTGCAGTCAGGCTTTGAACTTGCAGAC3'. The PCR was carried out using digoxigenin-labeled uridine triphosphate (DIG-labeled UTP) (Roche Diagnostics, Mannheim, Germany) under the following conditions: denaturing at 94℃ for 30 s, annealing at 57℃ for 30 s, and elongation at 72℃ for 1 min; 30 cycles were used.

Preparation of Reg I receptor cDNA probe

A Reg I receptor cDNA was prepared using double digestion of pCIneo-Reg I receptor cDNA plasmid [10] with *Hin*dⅢ and *Not* I. Electrophoresis of the digestion complex was performed on 8 g/L agarose gel, the receptor band cut from the gel, and the cDNA extracted from the gel using the QIAEX Ⅱ agarose gel extraction protocol (Qiagen, Valencia, CA). The reg I receptor cDNA fragment was labeled with digoxigenin (DIG) using the DIG High Prime DNA Labeling and Detection Starter Kit Ⅱ (Boehringer Mannheim, Germany) as per manufacturers' protocol.

Northern blot

RNA was extracted from rat pancreatic and intestinal tissue using Trizol reagent (Gibco, Life Technologies, Rockville, MD). RNA extraction was performed with 1-bromo-3-chloropropane. RNA concentrations were determined by absorbance measurements at 260 nm, and the integrity confirmed by polyacrylamide gel electrophoresis. Equal amounts (20 μg) of total RNA were then used for all Northern blots. The membrane was equilibrated in 10 × saline-sodium citrate (SSC) buffer for 5 min. The RNA samples were prepared as follows: 50 μL TE buffer (TE: 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0), 20 μL of 37 g/L formaldehyde, 30 μL of 20 × SSC, and 20 μg RNA. The samples were heated at 60℃ for 15 min and immediately chilled on ice for 3 min. Two hundred microliters of 10 × SSC were then added to each tube. The samples were applied to the nylon membrane using a Northern slot blot apparatus. Each well was washed with 500 μL SSC. The membrane with RNA was UV cross-linked and placed in 50 mL of DIG pre-hybridization solution at 48℃ for 6 h. Hybridization with the DIG-labeled reg I receptor cDNA probe was carried out at 58℃ for 12 h. After washing and blocking, the membrane was incubated in a 1:10000 dilution of anti-DIG-alkaline phosphatase conjugate antibody solution at room temperature for 30 min. CSPD solution (Boehringer Mannheim) was used for detection. Gels were photographed using Polaroid 667 film and digitized using an Epson 636 scanner. Band density analysis was performed using the public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/) to determine the quantity of nucleic acid product. To account for differences in the amounts of starting RNA between samples, the density of each reg band was normalized to that of the β-actin band for the same sample. Samples were run in duplicate and data are expressed as mean optical densitometric (OD) measurements ± SE.

Western blot on tissue homogenate to determine reg I levels

The tissue was homogenized in buffer containing 125

mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L EDTA, 5 mmol/L PIPES-Tris (pH 6.7), and 1 tablet of protease inhibitor cocktail per 50 mL of buffer. Total protein concentrations were determined using Bio-Rad protein assay. Electrophoresis was performed using equal amounts of total protein (10 μg/well) in a 100 g/L sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The electrophoresis was carried out at 80 V for the first 15 min and then at 130 V for 45 min in SDS electrophoresis buffer. The protein was transferred to nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) at 100 V for 1 h. The membrane was blocked overnight in 50 mL/L milk. A dilution of 1:5000 was used for the mouse anti-reg I mAb and incubated for 1 h with the membrane. After washing with T-TBS, the membrane was incubated for 45 min with a 1:2000 dilution of horse radish peroxidase-linked anti-mouse IgG. Detection was done using the ECL Western Blotting Detection System (Amersham Pharmacia, Piscataway, NJ). Human Reg 1 (hReg1) (10 μg) was used as a positive antibody control.

ELISA on serum samples to determine reg I *concentration*

Each well of the microtiter plate was coated with a mixture of 50 μL serum and 50 μL of coating buffer (20 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$). Serial dilutions of human reg I were used as standards. The wells were coated overnight, followed by blocking with a 3% BSA solution for 4 h. The mouse reg I mAb was diluted 1:1000 and incubated for 1 h[12,13]. A dilution of 1:1000 was used for the secondary antibody and incubated for 45 min. Detection was performed by adding 100 μL of detection buffer (4.84 mL of 0.05 citric acid, 5.14 mL of 0.1 Na_2HPO_4, 30 μL of 300 mL/L peroxide, and 1 tablet of o-phenylenediamine) and measuring absorbance at 450 nm.

Development of anti-receptor antibodies

Two non-overlapping peptide sequences were identified that were contained in the extracellular domain of the Reg I receptor[10] with predicted antigenicity and directly flanking specific amino acids thought to directly bind to Reg I (phage display experiment, unpublished data). PAELEKQLYSLPHWRTDC and RLLPEKDDAGLPPPKATRGC were synthesized by solid phase synthesis (Biomolecules Midwest Inc., St. Louis, MO), purified by reverse phase high performance liquid chromatography (HPLC), and characterized by Mass Spectrometry. Peptides were coupled to KLH and used for immunization of rabbits. Rabbits were boosted at monthly intervals and bled 10-14 d after each boost. Antibody titer was determined in a microtiter plate ELISA using the conjugated peptide as the immobilized antigen. The immunoglobulin fraction was isolated using ImmunoPure (A/G) IgG purification system (Pierce, Rockford, IL) and the final antibody concentration was determined with the BCA Assay (Pierce).

Reg I *receptor immunohistochemistry*

Paraffin embedded sections were treated with Ficin (1:100 dilution, Sigma, St. Louis, MO) for 30 min at room temperature, washed, and were then treated with Avidin/Biotin blocking reagent (Vector Labs, Burlingame, CA) for 20 min. Slides were subsequently treated with a Protein Block Reagent (Dako, Carpenteria, CA) and incubated with primary antibodies overnight at 4℃. Goat anti-rabbit biotinylated IgG secondary antibody was applied for 1 h at room temperature (1:1500, NEN Life Science, Boston, MA). Endogenous peroxidase activity was quenched by treatment with 10 mL/L hydrogen peroxide/PBS and sections were then incubated with streptavidin-horseradish peroxidase (SA-HRP) (1:1000 dilution, Dako) for 30 min. For receptor experiments, tyramide amplification was applied for 3 min (NEN Life Science, Boston, MA). Slides were extensively rinsed and again incubated with SA-HRP (Dako, 1:1000) for 30 min at room temperature. Slides were developed in DAB (Sigma, St. Louis, MO) and counterstained with hematoxylin (Richard Allan, Kalamazoo, MI).

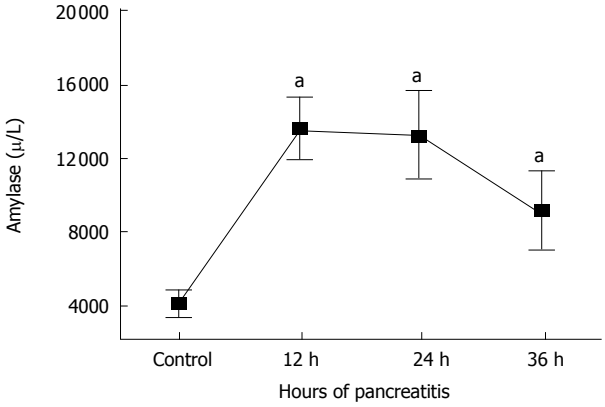

Figure 1 Serum amylase activity in pancreatitis rats (12, 24, 36 h) *vs* sham operated and normal controls. $^aP < 0.05$.

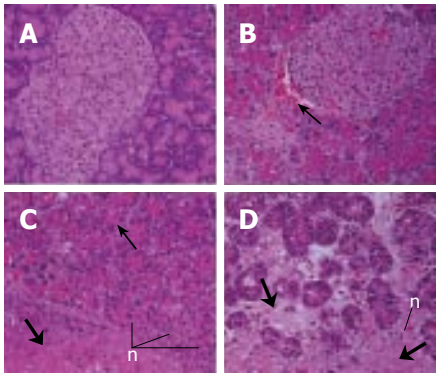

Figure 2 HE stain of normal rat pancreas (**A**) and after taurocholate injection at 12, 24, and 36 h (**B**, **C**, and **D**). Induction of pancreatitis resulted in hemorrhage (thin arrows), infiltration of neutrophils ("n"), and necrosis (thick arrows).

Statistical analysis

Data were expressed as means ± SE. Student's *t* test was used for analysis. $P < 0.05$ was taken as significant.

RESULTS

The presence of acute necrotizing pancreatitis in taurocholate-treated rats was confirmed by serum amylase activity and histology (Figures 1 and 2). As demonstrated in Figure 1 serum amylase levels at 12, 24, and 36 h were elevated ($P < 0.05$) when compared to both sham operated and normal control rats. Figure 1 demonstrates a rapid increase

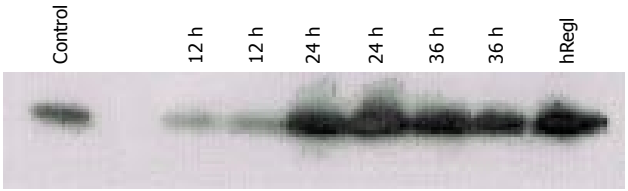

Figure 3 Slot blot analysis with anti-reg I antibody of total protein (10 μg/well) from normal rat pancreas and rat pancreas after induction of acute pancreatitis. Reg I protein levels in tissue increased after 24 h of pancreatitis. Human Reg I (hReg1) is shown as a loading control as described in MATERIALS AND METHODS.

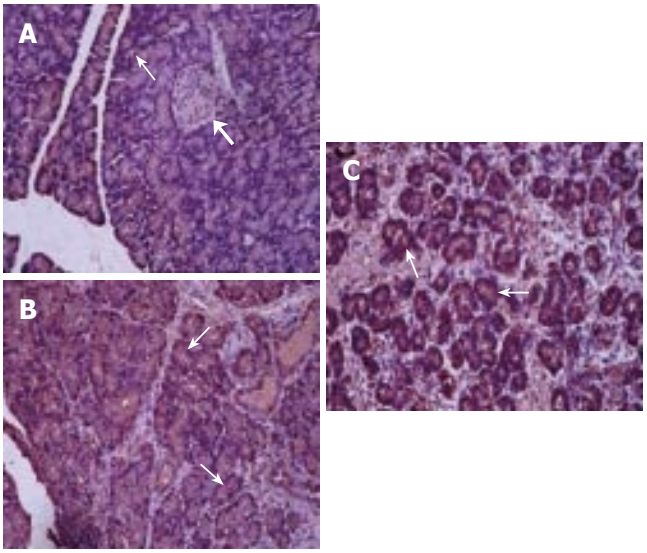

Figure 4 Immunohistochemical staining of normal rat pancreas with anti-reg antibody (**A**) and of rat pancreas after 24 and 36 h of pancreatitis (**B**, **C**). There is a marked increase in reg I levels in acinar cells of the pancreas after pancreatitis. Stained acinar cells are marked with thin arrows, compared with lack of staining in Islet cells (thick arrow).

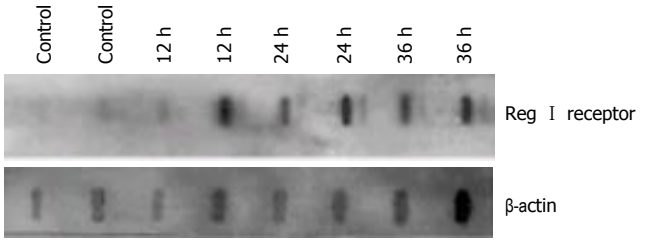

Figure 5 Slot blot of reg I receptor during pancreatitis showed an increase in reg I receptor mRNA expression after induction of pancreatitis. The density of each reg band was normalized to that of β-actin band for the same sample as described in MATERIALS AND METHODS.

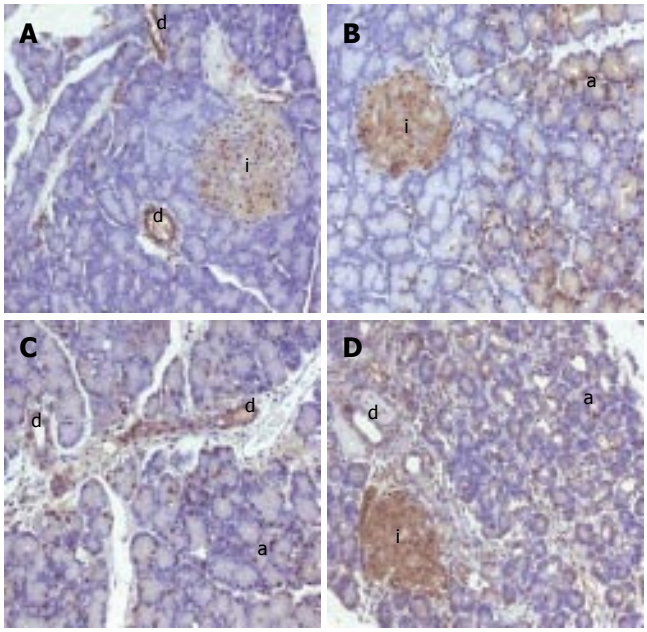

Figure 6 Immunohistochemical staining of pancreata from normal (**A**) and experimental pancreatitis (**B**-**D**) rats. There is an induction of reg I receptor expression in the acinar cells (a) and islets (i), and maintenance of reg I receptor expression in ductal cells (d) with pancreatitis.

in amylase levels at 12 h with a gradual decline towards normal levels at 24 and 36 h. Figure 2 demonstrates histopathological worsening of pancreatitis in the pancreatic parenchyma after sodium taurocholate treatment as evidenced by hemorrhage (Figures 2 B, C) and necrosis (Figure 2D) when compared with control groups (Figure 2A).

Western blot analysis of pancreatic tissue showed a single band at 15-17 ku using a monoclonal antibody and demonstrated an increase in reg I protein in the 24 and 36 h pancreatitis groups compared to controls, although at 12 h, there was an initial decrease in reg I protein (Figure 3). Histologic examination showed that this appeared localized in the pancreatic acinar cells (Figure 4). Serum reg I protein levels were not significantly different in pancreatitis (0.43 ± 0.13 mg/L) when compared with sham-operated and normal controls (0.38 ± 0.15 mg/L) (data not shown).

Northern/slot blot analysis demonstrated a single band corresponding to the Reg I receptor mRNA and showed an increase in reg I receptor RNA expression in pancreatic tissue from the experimental pancreatitis groups compared to controls (Figure 5). Mean absorbance (A) ± SE of reg I receptor signals on Northern/slot blot for control, 12, 24, and 36 h of pancreatitis were 24 ± 6, 71 ± 15, 66 ± 26, and 75 ± 20 (all were significantly increased compared to control, $P < 0.05$). Immunohistochemical staining with anti-reg I receptor antibody showed that although staining persisted in the ductal population, there was significant induction of receptor protein in both the islets and acinar cells (Figure 6).

DISCUSSION

Reg I is mitogenic to ductal and β-cells likely through the induction of the MAP kinase p38 pathway[14]. This has been shown to be true in both cell culture and animal models of pancreatectomized rats. The data presented support the hypothesis that reg I is involved in regeneration after injury to the pancreas in the form of a sodium taurocholate-induced acute necrotizing pancreatitis. The pancreatitic rats demonstrated an increase in reg I protein levels in pancreatic tissue after 24 h of pancreatitis. In the acutely inflamed pancreas it appeared that reg I protein was overproduced in the acinar cell population. This is consistent with the findings of others in both acute and chronic forms of pancreatitis[15]. Although many genes are

differentially regulated in inflammatory states, microarray data by us and others have demonstrated increased Reg expression in experimental pancreatitis[16,17]. Interestingly, serum reg I protein levels did not increase in parallel with acinar reg I RNA expression levels[13]. This may be due to differences in cellular RNA turnover prior to stabilization of protein levels in serum, possibly through unidentified reg I binding proteins. Similar discord between protein and gene expression has been found in other systems[18].

Baseline expression of reg I receptor is present in pancreatic ductal and beta cell lines, but not in acinar cell lines[10,19]. These cells respond to reg I protein by proliferating, as measured by thymidine incorporation. Cells transfected with the receptor respond to reg I in a similar fashion[10]. Acinar cells, which typically do not grow in response to reg I, do not express the receptor. It is therefore likely that the expression of the receptor is linked to the activity of cellular mitogenesis, and may be important in the proliferative response of pancreatic regeneration.

We found that pancreatitis significantly induced the expression of reg I receptor RNA in pancreatic tissue compared with baseline level. Immunohistochemical analysis with a polyclonal antibody to the reg I receptor localized the induced protein to the acinar cells and islets, and was only mildly present in ductal cells. This pattern of induction was in sharp contrast to the increase in reg I which was initially induced primarily in the acinar cells. While ductal cells typically proliferate after pancreatitis[20], the additional induction of the protein in islets and acinar cells likely means that they are becoming responsive to the reg I protein which is being produced and secreted by the acinar cells.

Our present data suggest that increased reg I protein and receptor are important in the response of the pancreas to injury. Specifically, reg I may exert its mitogenic effect on all the cell populations after injury. It likely works in a paracrine fashion, since it is produced and secreted from acinar cells. The induction of the receptor after injury in the acinar cells and islets and its maintenance of expression in the ductal cells may allow the protein to enhance its regenerative effect.

Another new protein induced in pancreatitis is reg Ⅲ, or pancreatitis associated protein (PAP). Seventy percent homologous to reg I, the pancreatic RNA and protein levels are increased even more dramatically in pancreatitis, and PAP protein is also increased in serum [21,22]. We showed that the bovine form of reg Ⅲ is mitogenic to pancreatic β- and ductal cells as well[3]. It is possible that reg Ⅲ protein interacts with the reg I receptor which may provide an important pathway for regeneration after injury.

Future experiments need to determine whether blocking the receptor will block the regenerative effect of reg I, and whether other members of the reg family, in particular reg Ⅲ, can stimulate the receptor as well.

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CLINICAL RESEARCH

Ultrasonographic study of mechanosensory properties in human esophagus during mechanical distension

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Abstract

AIM: To study the esophageal geometry and mechanosensation using endoscopic ultrasonography during volume-controlled ramp distensions in the distal esophagus.

METHODS: Twelve healthy volunteers underwent distension of a bag. During distension up to moderate pain the sensory intensity was assessed on a visual analogue scale (VAS). The esophageal deformation in terms of multidimensional stretch ratios and strains was calculated at different volumes and VAS levels. Distensions were done before and during administration of the anticholinergic drug butylscopolamine.

RESULTS: The stimulus-response (volume-VAS) curve did not differ without or with the administration of butylscopolamine. Analysis of stretch ratios demonstrated tensile stretch in circumferential direction, compression in radial direction and a small tensile stretch in longitudinal direction. A strain gradient existed throughout the esophageal wall with the largest circumferential deformation at the mucosal surface. The sensation intensity increased exponentially as function of the strains.

CONCLUSION: The method provides information of esophageal deformation gradients that correlate to the sensation intensity. Hence, it can be used to study mechanosensation in the human esophagus. Further studies are needed to determine the exact deformation stimulus for the esophageal mechanoreceptors.

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Key words: Deformation analysis; Esophagus; Experimental pain; Ultrasonography

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INTRODUCTION

Acute and chronic pain in the upper gastrointestinal tract is a severe and frequent health care problem in terms of human suffering and enormous economic implications for the health care system[1]. Thorough understandings of visceral pain mechanisms are necessary for developing diagnostic tools and optimal treatments. However, pain and related symptoms are difficult to study due to the heterogeneity of patients and confounders related to clinical pain[2].

Human experimental pain models can be used as tools for better characterization and understanding of pain mechanisms. In recent years studies have been focused on mechanical distension[3-5]. However, many experimental pain studies of hollow visceral organs have reached erroneous conclusions since the pressure, volume and wall tension are proxies of the deformation in the vicinity of mechanoreceptors and therefore only show indirect degree of association with the evoked responses. It has been demonstrated in several parts of the digestive tract including the esophagus that deformation (circumferential strain) is more closely associated with the distension-induced sensory responses than the distension pressure, volume or wall tension[6-8]. Calculation of the strain, however, demands advanced methods. Recently, endoscopic ultrasonography has been used to obtain information about the geometry of the esophagus including wall and layer thicknesses for the purpose of computing wall tension, stress and strain during swallowing[9-12]. However, none of these studies has carefully evaluated the sensory properties during esophageal distension.

In the present study, endoscopic ultrasonography was combined with controlled esophageal distension and sensory assessment to obtain information about the mecha-

nosensation. Since deformation is considered the most important parameter for mechanosensation[6-8], we measured the needed geometric variables and computed multidimensional stretch ratios and strains in order to obtain new information about the relationship between stretch and evoked pain.

MATERIALS AND METHODS

Subjects

Twelve healthy subjects, 6 males and 6 females, median age 38 years (range 22-67 years) recruited from the hospital and university staff, volunteered to participate in the study. They did not suffer from previous or current gastrointestinal disease and had no gastrointestinal complaints. The volunteers did not take any drugs and abuse alcohol. The regional ethics committee approved the study protocol and the experiments were conducted in accordance with the revised Declaration of Helsinki. All volunteers were asked to give a written informed consent to participate in the experiment.

Probe design

The probe was constructed from a thin flexible tube and equipped at the end with a highly compliant latex balloon. The tube was connected to an infusion pump (Type 111, Ole Dich Instrument makers, Hvidovre, Denmark). Saline heated to body temperature was infused into the balloon at the rate of 25 mL per min. The 6-cm long balloon was made of natural rubber with a thickness of 0.2 mm. In the deflated state the largest transversal diameter of the balloon was 10 mm. The shape of the balloon became almost spherical when inflated. An ultrasonographic miniprobe was advanced through the probe lumen and the transducer was placed inside the balloon. The ultrasound system used a rotating 20-MHz transducer (model UM-3R; Olympus, Japan). The ultrasound transducer produced a real time 360-degree cross-sectional image of the esophagus.

Protocol

The subjects were fasted at least for 6 h. Studies were performed with the subjects in half supine position. The probe was lubricated and inserted through the nostrils. The tip of the probe was first advanced into the stomach. The lower esophageal sphincter (LES) was identified by pull-through manometry as a zone of resting pressure that decreases with swallowing. The balloon was placed 8 cm proximal to LES and the probe was fixed to the nose to prevent migration of the assembly during the study.

Distensions were done to precondition the tissue and served as learning sessions to facilitate the sensory rating. Since preconditioning is necessary to obtain reproducible results[4], three distensions using a constant infusion rate of 25 mL/min were done until the pain threshold was reached (5 on the visual analogue scale (VAS). After the preconditioning distensions, two more distensions to 7 on the VAS (moderate pain level) were done. Then a bolus of 20 mg butylscopolamine was given intravenously to decrease distension-induced secondary smooth muscle contractions. The balloon was again inflated twice using an infusion rate of 25 mL/min until 7 on the VAS was reached. The balloon was emptied for 2 min between the distensions. None of the volunteers experienced significant side effects due to the anti-cholinergic action of the drug. The total investigation time never exceeded 1.5 h.

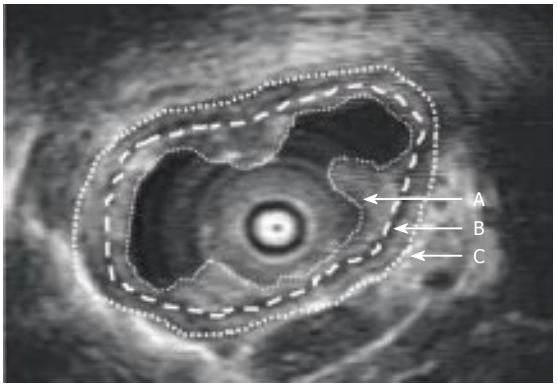

Figure 1 Transversal ultrasonographic image of the non-distended esophagus. The stippled lines indicate manual tracings of the mucosal inner surface (A) and the inner (B) and outer (C) lining of the combined muscle layers.

Sensory assessment

The sensory intensity was assessed continuously during the mechanical distension by using an electronic VAS-meter (GMC-Medical, Hornslet, Denmark). The VAS data were amplified and analog-to-digital converted at a sampling rate of 10 Hz using the Openlab data acquisition system (GMC-Medical, Hornslet, Denmark). The digitized data were stored on a PC for later analysis.

Before commencement of the visceral mechanical stimulation, the subjects were trained in using the VAS. The sensations were produced by applying deep pressure in muscles of the right forearm. The intensity of non-painful stimuli was scored up to 5 on the VAS. Key anchor words to describe the sensations were: 1 = vague perception of mild sensation; 2 = definite perception of mild sensation; 3 = vague perception of moderate sensation; 4 = definite perception of moderate sensation; 5 = pain threshold. For the painful sensations the patients used the scale from 5-7 anchored at 6 = slight pain and 7 = moderate pain[2].

Data analysis

Ultrasonographic images of the esophagus were recorded in real time during the studies using a super-VHS videotape recorder. The videotape recordings were captured in video streaming 120 + MB/s at a frame rate of 25 s^{-1} and a resolution 756 × 576 by a high performance video grabber (I-Color, Image House A/S Copenhagen DK). All image measurements and calculations were performed off-line using an image analysis software package (Scion Image Beta 4.02 Win, Maryland). We selected one reference image at each pain intensity level from the distensions after preconditioning the tissue. The images were selected at points where contractions were not present. The mucosa and muscle layers were outlined manually (Figure 1). Once outlined, the software program automatically calculated the circumference and the area of the layers. The layer

thickness was computed from the circumferential length and area.

The steady state pressure, diameters, CSA and wall thickness values at each volume distension step were used for the computation of stretch ratios and strains.

The circumferential Cauchy strain ε was computed as the fractional change in circumference[3].

$$\varepsilon = \frac{c - c_0}{c_0} \quad (1)$$

where C is the circumference obtained at the serosal or mucosal surfaces or between layers at various degrees of distension, C_0 is the reference circumference obtained at unloaded conditions immediately prior to a distension. The strain in radial direction could be computed from the measured wall thickness h as

$$\varepsilon = \frac{h - h_0}{h_0} \quad (2)$$

where h_0 is the reference thickness obtained at unloaded conditions immediately prior to a distension. In a similar way the strain could be computed for the individual wall layers.

In a mechanical analysis it may also be convenient to use the stretch ratio as a measure of deformation. The stretch ratio λ was determined for both the circumferential (θ), radial (r) and longitudinal (z) direction. The stretch ratio is defined as the length at loaded conditions (L) divided by a reference length at unloaded conditions (L_0).

$$\lambda = \frac{L}{L_0} \quad (3)$$

Thus the stretch ratio $\lambda = \varepsilon + 1$. For circumferential and radial direction the stretch ratios could be determined from the experiments using the same principle as for the strain calculation. The stretch ratio in longitudinal direction λ_z was determined from the two other stretch ratios by using the law of mass conservation. Hence, $\lambda_z = 1/\lambda_\theta\lambda_r$[3,4,13].

Statistical analysis

The results were expressed as mean ± SE. At various volumes and VAS levels 1-7, the corresponding stretch ratios before and during administration of butylscopolamine were determined. Two-way-ANOVA was used for statistics. $P < 0.05$ was considered statistically significant.

RESULTS

Ultrasonographic and VAS data were obtained in all subjects. The distensions were done until moderate pain (VAS = 7) was experienced except in one subject in whom the distension was done only until the pain threshold (VAS = 5) was reached. Three subjects repeatedly got involuntary long lasting contractions in the esophagus after the pain threshold was reached. Butylscopolamine greatly reduced these contractions. One subject reported autonomic side-effects during the distensions manifested as tachycardia, nausea and sweating.

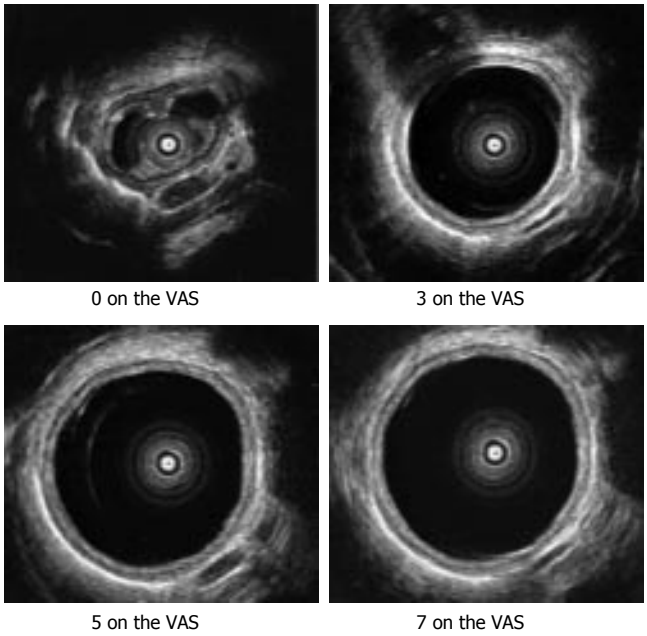

Figure 2 Transversal ultrasonographic images of the esophagus at different sensory perception levels. The esophagus is fairly circular at 3 on the visual analogue scale. The numbers refer to the sensory intensity at a visual analog scale with 5 as the pain threshold.

The preconditioning stimuli resulted in reproducible mechanical distensions and sensory-motor responses. Figure 2 shows the representative ultrasound images obtained at different VAS levels. The mean thickness of the mucosa and muscle layer in the resting state was 1.4 ± 0.02 mm and 2.4 ± 0.03 mm respectively. The esophageal folds almost disappeared at VAS level of 3 or higher where the geometry could be approximated as circular. The interface between the inner circular and outer longitudinal muscular layer could be visualized as a thin echogenic layer only at low volumes in the balloon. This layer was not consistently visible in the subjects at VAS levels of 2 or higher (Figure 2) and therefore could not be used in the strain analysis.

We found no difference in the volume-VAS data before and during butylscopolamine infusion (Figure 3-top, $F = 2.50$, $P = 0.12$). Consequently we used the data obtained during butylscopolamine infusion for the further analysis since contractions to some degree would bias the readings. Thus, the increased VAS primarily would be due to stretch of the wall rather than the active stress generated by the muscle tissue. Figure 3 also shows the circumferential, radial and longitudinal stretch ratios as function of volume and sensory intensity on the VAS for the mucosa and main muscle layers. In both layers, the circumferential deformation was tensile, whereas the radial deformation was compressive due to the decrease in layer thickness during the distensions. The computed longitudinal stretch ratio was tensile but less than the circumferential stretch ratio. Furthermore, the longitudinal stretch ratio did not increase further after 1 was reported on the VAS or a volume of 10 mL was reached. At the pain threshold (VAS 5) the mean circumferential, radial and longitudinal stretch ratios in the muscle layer were 1.7 ± 0.1, 0.5 ± 0.04 and 1.2 ± 0.07, respectively.

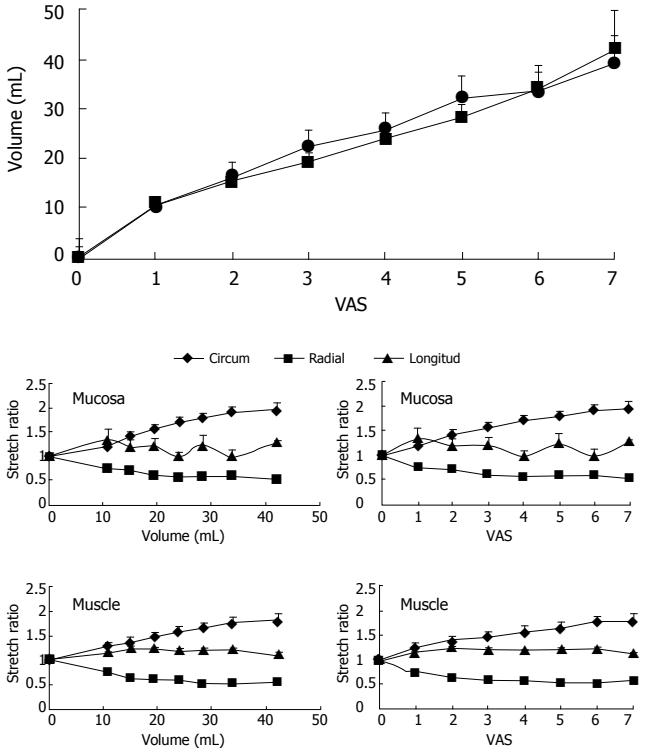

Figure 3 Illustration of the relation between volume and sensory intensity on the visual analogue scale (VAS) with 5 as the pain threshold (top graph). Since there was no difference in the curves obtained (triangles) before and during (squares) infusion of butylscopolamine, data obtained during butylscopolamine are given in this paper. The four figures show the circumferential, radial and longitudinal stretch ratios as function of volume and VAS in the mucosa and muscle layers (the circumferential data are from the mucosal surface and the outer muscle surface).

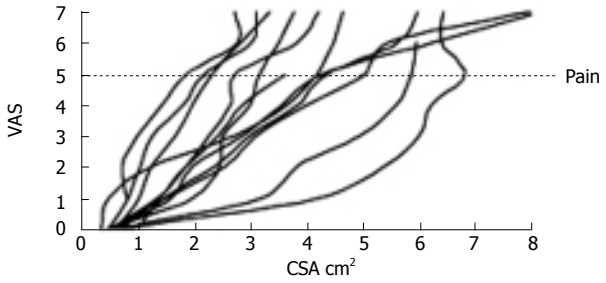

Figure 4 Sensory intensity on the visual analogue scale (VAS) with 5 as the pain threshold as function of the luminal cross-sectional area (CSA). A large variation was found in volunteers, which speaks in favor of using non-dimensional measures as the stretch ratio.

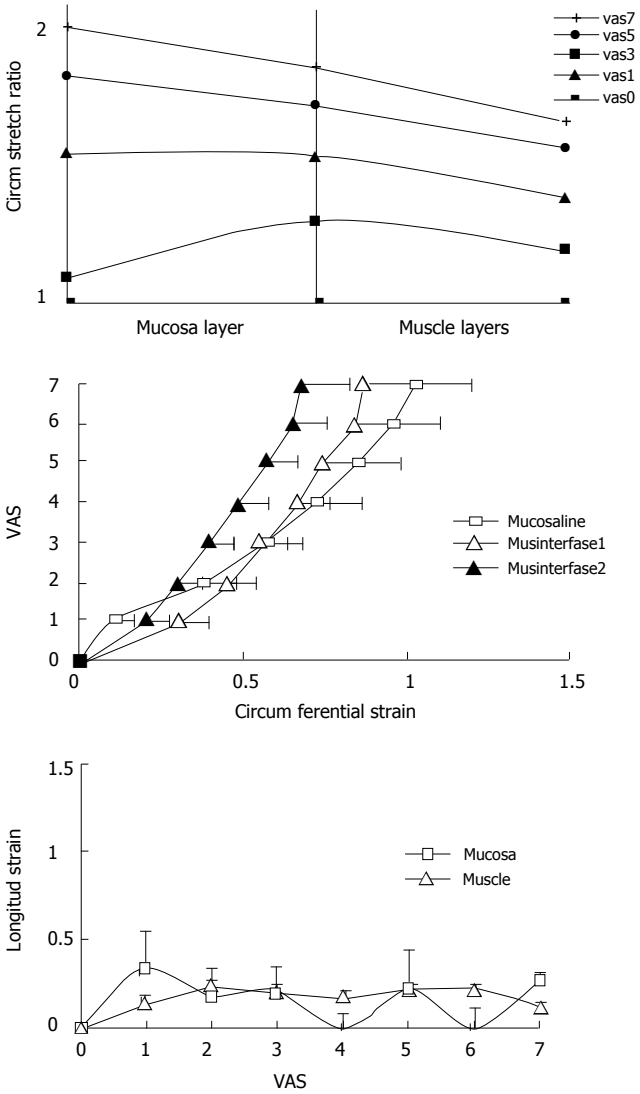

Figure 5 The top graph shows the circumferential stretch ratio at the mucosal surface (left), the interface between the submucosa and circumferential muscle layer (middle) and the serosal surface (right). The middle graph shows the same data in terms of the circumferential strain and sensory intensity on the visual analogue scale (VAS). There was a strain gradient throughout the esophageal wall with the largest deformation in the mucosa. The bottom graph shows the longitudinal strain as function of the sensory level. No increase in strain was seen during sensory intensity was increased. For convenience the data from all VAS are not shown.

The sensory response showed a large variation between the volunteers. Figure 4 shows the sensation as function of luminal cross-sectional area (CSA). The rather large inter-individual variation speaks in favor of using non-dimensional measures such as stretch ratios rather than the volume or CSA. Figure 5 shows the circumferential stretch level at the mucosa and the inner and outer muscle layers. The circumferential deformation at the mucosal surface was larger than that at the outer muscle layer at high sensory levels. However, at low VAS levels the mucosal deformation was small, which was due to unfolding rather than stretch of the mucosa initially during the distensions. A strain gradient existed through the esophageal wall with the largest deformation in the mucosa and the sensation increased exponentially as function of the strain in both the mucosa and muscle layers (Figure 5). Longitudinal strain during distension seemed not to cause the sensation as the longitudinal strain did not increase further after VAS 1 (Figure 5 bottom). To evaluate further whether radial or circumferential strains were most important, we plotted the dispersion (SE/mean) of the radial and circumferential stretch ratio data from Figure 3. The circumferential stretch ratios in the muscle and mucosa layers showed the smallest dispersion (Figure 6).

DISCUSSION

The function of the esophagus is mechanical to a large extent. Swallowed contents are propelled through the esoph-

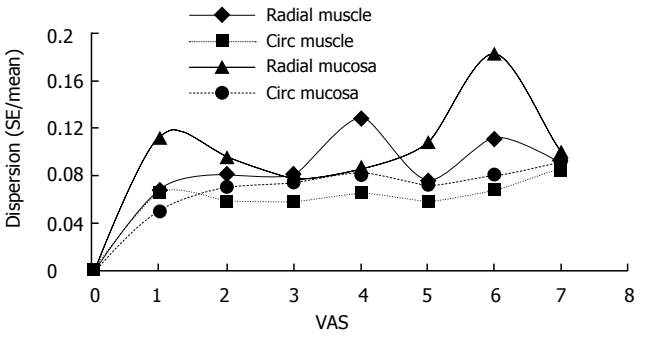

Figure 6 Dispersion (SE/mean) of the circumferential and radial strains in the mucosa and muscle. The dispersion was the lowest in circumferential direction.

agus into the stomach where the digestion starts. Methods traditionally used for clinical or basic investigations of the esophagus are endoscopy, manometry, pH-metry and radiographic examinations. Although these methods provide important data on esophageal motor function, little attention has been paid to the biomechanical and mechanosensory properties of the wall and determination of the wall deformation. During recent years ultrasonography has gained interest in studying gastrointestinal (GI) physiology and in clinical evaluation of the esophagus. In the current study, the stretch was tensile in circumferential direction, compressive in radial direction and tensile to a small degree in the longitudinal direction. A strain gradient existed throughout the esophageal wall with the largest circumferential deformation at the mucosal surface. The sensation intensity increased exponentially as function of the strains and the esophageal deformation gradients correlated to the sensation intensity.

Endoscopic ultrasonography is a non-invasive and safe method applicable in study of the esophagus. Two-dimensional B-mode ultrasonography has been utilized to assess the correlation between ultrasonographic images and histology[13] and to determine biomechanical properties of the esophagus *in vitro*[14]. Furthermore, Nicosia *et al*[10] have used endoscopic ultrasonography in humans to study esophageal longitudinal muscle shortening and Miller *et al*[11] have studied the correlation between wall changes and manometry during esophageal motility. Moreover, Pehlivanov *et al*[9] showed that muscle geometry is changed during contractions in volunteers and patients with esophageal spasms. None of these studies has paid attention to the sensation provoked by balloon distension. Thus, despite these developments and the fact that the mechanical wall properties are important for normal function of the esophagus, the mechanosensory properties of the esophagus *in vivo* are still largely unknown. In this study we therefore intended to gain a better understanding of esophageal deformation and mechanosensory function.

Methodological aspects

In distensible biological tubes, the circumferential wall strain is of interest because the tensile stress is the largest in that direction during distension[3,4,15]. The reasons for studying the stresses and strains in intact hollow organs are as follows: wall stress and strain data give valuable information on the elastic properties[4], mechanoreceptors do not respond directly to changes in pressure or volume but rather to changes in strain[2], it is important to differentiate active properties such as phasic contractions and smooth muscle tone from passive tissue properties in pharmacological and biomechanical studies[2,16,17], and intact organs maintain normal geometry in contrast to the tissue strips often used for length-tension measurements in physiological and pharmacological studies[15,18].

In this study, we aimed to develop a new method for investigation of the mechanosensory properties in the esophagus, mainly in the state where contractions did not confound the analysis. The method is based on distension of a bag placed in the lumen with concomitant measurement of the distension pressure and the antral geometry in a selected cross-sectional plane by endoscopic real-time B-mode ultrasound. The ramp protocol ensured that we studied the elastic properties rather than viscoelastic properties[4,15]. This ease the interpretation of the data since time-dependent confounders are significantly eliminated[4]. The administration of the antimuscarinic drug butylscopolamine allowed us to investigate the active and passive tissue behavior. Since the volume-VAS curve did not differ between the distensions before and during butylscopolamine administration, we only analyzed the distensions after administration of butylscopolamine. This gives data that are easier to interpret as there is no significant influence of esophageal contractions. Butylscopolamine may not abolish all contractions. Therefore we analyzed the data between contractions if they were present. Since stretch as well as contractions may elicit pain, we primarily looked into the stretch-dependent pain mechanisms in this study. It has been found in other tissues as in the cardiovascular system that the baroreceptors depend on strains in the vicinity of the receptors[15]. We have previously shown that strain is the most important biomechanical parameter for sensory responses in the gastrointestinal tract including the esophagus[6-8]. Thus, in this study we were only concerned with the deformation as a stimulus for the mechanoreceptors.

The ultrasonographic technique makes the derivation of strains in multidimensional directions possible from the geometric data or due to use of the mass conservation principle. The strain calculations are based on direct measurements rather than on geometric assumptions such as a circular cross-section and the thickness of the layers measured in our study, which are consistent with the data presented by others[10]. The fact that the lumen is not circular at low volumes makes it difficult to compute tension and stress based on Laplace's law[3,4,15], but the strain analysis does not depend on the geometry as long as the surfaces and interfaces between layers can be clearly defined. In most cases we did not have any problems in identifying the tissue layers. Thus the error due to these measurements is considered small. However, the ultrasound images only show the primary convolution (folds) but not secondary and tertiary convolutions of the mucosa. If higher-order convolutions were present, this could result in a minor underestimation of the calculated stretch in the mucosa and a larger inter-individual variation. Unfortunately, we could

not clearly outline the interface between the two muscle layers at VAS levels above 3. Hence, we omitted presenting data on this interface.

Biomechanical and mechanosensory aspects

In the present study, the deformation was the largest in the circumferential direction, despite the presence of mucosal folds the circumferential deformation decreased through the wall with the largest values at the mucosal surface, and the sensation increased exponentially as function of the circumferential strain.

Gastrointestinal symptoms are often associated with disturbances in motility and sensory function. Several studies have attempted to investigate these properties by means of balloon distension[19-21]. Unfortunately, the primary mechanism for symptoms elicited by GI distension remains unclear. It is well known that distension of the gastrointestinal tract elicits reflex-mediated inhibition and stimulation of motility via intrinsic or extrinsic neural circuits and induces visceral perceptions such as pain[21]. Previous studies demonstrated that mechanoreceptors located in the intestinal wall play an important role in the stimulus-response function[22-26]. It is, however, a common mistake to believe that mechanoreceptors are sensitive to variation in pressure, volume or tension. A large variation in the perception has been found in various studies and species[27-29], suggesting that pressure is not the direct stimulus. The dogma that the mechanoreceptors are tension-sensitive receptors lying in-series or in-parallel with the muscle cells has also been prevalent for many years[30]. This concept is borrowed from striated muscle physiologists and should yet be regarded as a working hypothesis, since no clear evidence supports it in GI studies. It is basically a uniaxial model that does not account for more complex biomechanical properties such as distribution of the deformation field and that different receptor populations may exist. There seems to be no evidence that the receptor is dependent of tension rather than strain. Instead, the receptors are stimulated by deformations acting on the intestinal wall due to changes in the transmural pressure[31]. Thus, the mechanical distension stimulus and the biomechanical tissue properties must be taken into account in studies of the sensory-motor function in the esophagus.

It is well known that the passive elastic behavior of biological tissues is exponential[3,4,15]. This mechanical feature protects the tissue against over-distension and damage at high luminal pressure loads while distending easily and facilitating flow in the physiological pressure range. In arteries, it has been demonstrated that collagen bears circumferential loads at high stress levels[32,33]. As gastrointestinal tissue is rich in collagen[34], it is likely that collagen is a major determinant of the passive mechanical properties. Though we did not evaluate the elastic properties in terms of stress-strain relations, the fact that the strain-VAS curve is exponential indicates that the exponential properties play a role in protecting tissue against high stress.

Our results may shed some light on the discussion regarding the visceral sensation receptors in human beings. The data suggest that at low loads the deformation occurs mainly in the muscle layer, whereas at higher loads, the largest deformation occurs at the mucosal surface. Furthermore, the lowest dispersion has been found for the circumferential stretch ratio in the muscle layer. Though this analysis may be biased by measurement of errors for the two directions, the analysis may reveal that the most important mechanoreceptors are located in the muscle layer where they sense circumferential deformation. This view is also supported by other data showing that the circumferential deformation in the mucosa is much smaller than in the muscle layer at low loads, where the mucosa merely unfolds rather than stretches. However, the differences are small because the deformations in various directions depend on each other (due to the mass conservation principle) and require further analysis.

Conclusions and future clinical applications

We have developed a new method to study the strain-dependency of esophageal mechanoreceptors. The data point in the direction of a strain receptor sensitive to circumferential deformation in the muscle layer. The new test can be used to test drugs on esophageal mechanosensory function, and improve our understanding of the pathophysiology underlying the symptom induction in patients with pain and other symptoms relating to the esophagus.

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CLINICAL RESEARCH

Computed tomographic differentiation between alcoholic and gallstone pancreatitis: Significance of distribution of infiltration or fluid collection

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Abstract

AIM: To evaluate the usefulness of various computed tomography (CT) findings including distribution of infiltration or fluid collection in differentiating the major etiologies of acute pancreatitis.

METHODS: We reviewed 75 relatively severe cases of acute pancreatitis of alcoholic (n = 43) or biliary stone (n = 32) etiology having infiltration or fluid collection on CT. We compared the pancreatic size, CT grading, presence or absence of biliary calculi, and dilatation of pancreatic or bile duct. We also evaluated degree and the distribution of infiltration and fluid collection in each group.

RESULTS: The sizes of pancreas were not different between alcohol group and stone group. Alcohol group showed higher CT grading than stone group (P < 0.05). Presence of biliary stone and duct dilatation was statistically significant in differentiating etiology (P < 0.05). Alcohol group showed significantly prominent peripancreatic pathology than stone group only in left peritoneal compartment (P = 0.020).

CONCLUSION: Alcoholic pancreatitis tends to form more prominent peripancreatic changes than gallstone pancreatitis in relatively severe cases. This is evident on the anterior aspect of left abdomen. Although clinical history and some CT findings usually are a major determinant of the etiology, this pattern of peripancreatic pathology may have an ancillary role in determining the etiologies of acute pancreatitis in the equivocal cases.

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Key words: Pancreatitis; Pancreas; Computed tomography; Peritoneum; Fluid; Retroperitoneal space

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INTRODUCTION

Biliary stones and alcohol account for 70%-80% of all acute pancreatitis etiologies[1]. Differentiation between etiologies is of great importance because it can affect the further diagnostic and therapeutic strategies. Diagnosis and treatment of gallstone pancreatitis require an endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy, but it is not warranted in alcohol-induced pancreatitis[2]. The risks associated with ERCP outweigh any benefit unless the causative biliary stone is present.

Computed tomography (CT) findings associated with gallstone pancreatitis such as presence of stone in the biliary system have been well defined. Clinical information about biliary colic or alcohol abuse is also very useful in diagnosis. Causes of acute pancreatitis are often easily determined. However, some cases lacking medical background or the localization of stones or sludge make cause determination difficult. Although CT findings cannot be a sole determinant of therapy in individual cases, it can play a complementary role especially in indeterminate cases.

The purpose of this study was to evaluate the usefulness of CT findings associated with acute pancreatitis and to differentiate between the two major causes of acute pancreatitis. Special focus was given to the degree and distribution of peripancreatic infiltrations and fluid collection.

MATERIALS AND METHODS

Patients

Abdominal CT scans of 86 patients who had CT diagnosis of acute pancreatitis and were evaluated to have an infiltration or fluid collection in the abdominal cavity were studied retrospectively. All of them could be graded as C, D,

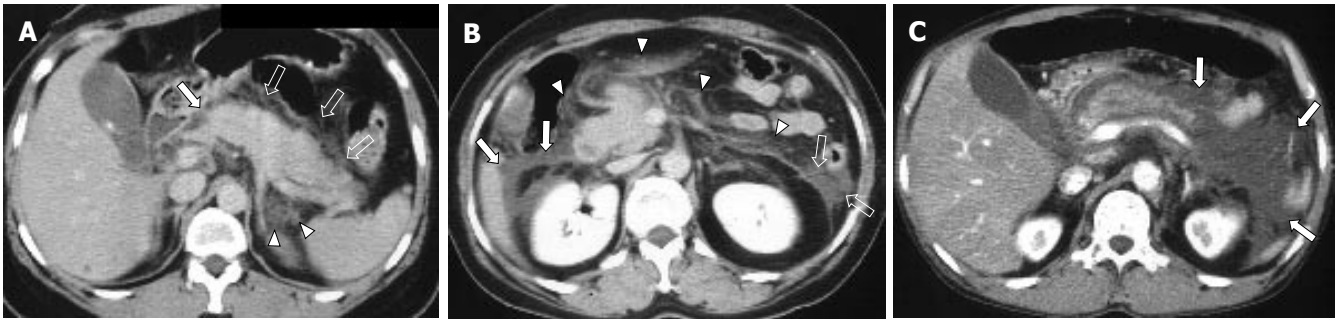

Figure 1 Peripancreatic infiltration scores (1-3). **A**: Score 1 (Irregular peripancreatic infiltration without fluid collection). CT showing irregular strands of peripancreatic fat infiltrations in the compartment of RP (arrow), LP (open arrows), and LSR (arrowhead); **B**: Score 2 (Peripancreatic fluid collection with no or equivocal degree of mass effect). CT showing multiple fluid collections without mass effect in the compartment of RSR (arrows) and LSR (open arrows). Score 1 infiltrations in the compartment of RP and LP (arrowheads) are also seen; **C**: Score 3 (Peripancreatic fluid collection with definite mass effect to adjacent organs). CT showing score 3 fluid collection in the LP and LSR compartments (arrows) displacing or compressing adjacent bowels.

and E by the system suggested by Balthazar *et al*[3]. Grade C was defined as pancreatitis with peripancreatic infiltration only. Grades D and E were defined as the disease with single and multiple peripancreatic fluid collections, respectively. Data were not consecutive but extracted randomly from our radiology department CT database. The population was composed of 53 male patients and 33 female patients with a mean age of 56.5 (range, 20-85) years. Patients were divided into an alcohol group and a stone group based upon their clinical diagnosis made by taking into account all possible factors including past medical history such as alcohol abuse or biliary colic, results of radiological studies (ultrasonography, CT, and ERCP), and laboratory data.

Of the 86 patients, eleven were excluded from the study because of unknown causes (n = 6), causes other than alcohol abuse or biliary stone (n = 3), traumatic pancreatitis (n = 1), L-asparaginase-induced pancreatitis (n = 1), pancreatitis due to pancreatic divisum (n = 1), and cases that were indeterminable between alcoholic and stone pancreatitis (n = 2) clinically. The remaining 75 patients were composed of 49 male patients and 26 female patients, with a mean age of 51.3 (range, 27-85) years. Forty-three out of 75 patients (57.3%) were classified as alcohol group, while 32 out of 75 (42.7%) were classified as stone group.

The Institutional Review Board of our hospital did not require approval for retrospective clinical study.

CT protocol

Abdomen CT scans were performed with one of the two helical scanners (Somatom Plus or Somatom Plus 4; Siemens, Erlangen, Germany). Helical CT images of pre-contrast and contrast-enhanced scans were acquired using an 8-mm collimation and 10-mm/s speed (Table 1). Images were reconstructed at an 8-mm interval. A voltage of 120 kVp and amperage of 210 mAs were used. One hundred mL of non-ionic contrast material (Iopromide, Ultravist-300; Schering AG, Berlingen, Germany) was administrated intravenously via the antecubital vein at a rate of 3 mL/s with a power injector (OP 100; Medrad, Pittsburgh, PA). Dual-phase contrast-enhancement scan technique was adopted with a delay time of 60-70 s and 200-220 s, respectively, in the majority of cases. Pancreatic phase scan with a delay time of 30-40 s was available only in a small portion of the patients (n = 13) because of lacking of the clinical diagnosis of acute pancreatitis before CT examination. At least one of the two or three phases covered an entire abdominal cavity from diaphragmatic dome to symphysis pubis.

Image analysis

Two abdominal radiologists analyzed the CT findings in consensus. In patients undergoing multiple follow-up CT scans, only the initial study was selected for imaging analysis. The antero-posterior and transverse diameters of pancreatic head, as well as the thickest dimension of body or tail of pancreas were measured. We evaluated the severity of disease at the initial abdominal CT using the CT grading system introduced by Balthazar *et al*[3]. We classified them into a three-point-scale, and converted C, D, and E to 1, 2, and 3. We investigated the presence or absence of calculus. Calculus was thought to be present when we saw high attenuation in the biliary tree including gallbladder on pre-contrast scan without measurement of Hounsfield unit. We also examined whether the pancreatic or bile duct was dilated or not. The criteria for abnormal duct dilatation were over 8 mm in diameter for common bile duct and 3 mm in diameter for pancreatic duct.

To assess the distribution of peripancreatic infiltration or fluid collection, the abdominal cavity was divided into 6 compartments, namely right peritoneal (RP) compartment, right superior retroperitoneal (RSR) compartment, right inferior retroperitoneal (RIR) compartment, left peritoneal (LP) compartment, left superior retroperitoneal (LSR) compartment, and left inferior retroperitoneal (LIR) compartment. 'Right' and 'left' compartments were divided by median line of the body traversing the umbilicus and the spinous process of vertebra. 'Peritoneal' and 'retroperitoneal' compartments were divided by already-established anatomical interface between peritoneum and retroperitoneum. Finally, 'superior' and 'inferior' compartments were compartmentalized by the level of lower pole of the left kidney. Degrees of infiltration or fluid collection in each compartment were evaluated with a four-point scale from 0 to 3 (0: no infiltration, 1: irregular infiltrative attenuation without fluid collection, 2: fluid collection with no or equivocal degree of mass effect on

Table 1 CT differentiation of the cause of acute pancreatitis between alcohol and stone group (mean ± SD)

Findings	Alcohol group (n = 43)	Stone group (n = 32)	P
Size of pancreas			
Anteroposterior diameter, Head	36.2 ± 6.7 mm	37.3 ± 6.6 mm	0.485
Transverse diameter, Head	30.7 ± 5.6 mm	31.7 ± 5.4mm	0.434
Thickness, Body and Tail	22.3 ± 5.2 mm	23.3 ± 5.3 mm	0.406
CT grading score	2.3 ± 0.8	1.9 ± 1.0	0.047[1]
Calculi in the biliary system	3/43 (7.0%)	22/32 (68.8%)	0.000[1]
Duct dilatation			
Pancreatic duct	4/43 (9.0%)	10/32 (31.3%)	0.033[1]
Bile duct	2/43 (4.7%)	19/32 (59.4%)	0.000[1]

[1]Data of statistical significance.

Table 2 Comparison of infiltration scores of each abdominal compartment between alcohol group and stone group (mean ± SD)

Compartment	Alcohol group (n = 43)	Stone group (n = 32)	P
RP	1.00 ± 0.82	0.91 ± 0.69	0.568
RSR	0.84 ± 0.87	0.94 ± 0.95	0.416
RIR	0.56 ± 0.85	0.56 ± 0.84	0.905
Total (right)	2.40 ± 2.16	2.41 ± 2.12	0.798
LP	1.67 ± 0.97	1.00 ± 0.92	0.020[1]
LSR	1.49 ± 0.88	1.13 ± 0.83	0.119
LIR	0.51 ± 0.28	0.83 ± 0.52	0.240
Total (left)	3.67 ± 2.20	2.41 ± 1.79	0.153

Abbreviations indicating compartments defined in the text.
[1]Data of statistical significance.

adjacent organ, 3: fluid collection with a definite mass effect on adjacent organ) (Figure 1). If major infiltration or fluid collection of one compartment slightly extended to another compartment, the minor lesion was disregarded. Pancreatic necrosis or pseudocyst was regarded and analyzed as a fluid collection. Infiltration scores of each abdominal compartment in one group were compared with those of the corresponding compartment of the other group. Student t-test (pancreatic size), likelihood ratio test (CT grading of acute pancreatitis, grading of infiltration and fluid collection), and Fisher's exact test (calculi and duct dilatation) with 95% confidence interval were used for the evaluation of statistical significance (SPSS for Windows 11.0, Chicago, IL).

RESULTS

The antero-posterior and transverse diameters of pancreatic head for the alcohol and stone group measured 36.2 ± 6.7 mm × 30.7 ± 5.6 mm and 37.3 ± 6.6 mm × 31.7 ± 5.4 mm, respectively. The thickness of pancreatic body or tail in great dimension was 22.3 ± 5.2 mm and 23.3 ± 5.3 mm, respectively. Differences in pancreas dimension between the alcohol and stone groups were not significant ($P > 0.05$, Student-t test).

Scores of alcohol and stone groups resulting from CT grading (converted from C, D, E to 1, 2, 3), were 2.33 ± 0.81, and 1.94 ± 0.95, respectively. Alcohol group showed more aggressive CT findings than stone group ($P = 0.047$, likelihood ratio test).

The alcohol group showed calculi in the biliary tree in 3 of 43 cases (7.0%), while 22 of 32 cases (68.8%) were calculus-positive in the stone group ($P = 0.000$, Fisher's exact test). However, only 5 of 22 stone-positive cases showed calculi in the distal common bile duct. Only 5 of 32 cases (15.6%) in stone group showed distal common bile duct calculi on CT scans. In the remaining cases of stone group, biliary calculi were located in the gallbladder (n = 11), intrahepatic duct (n = 1), common duct other than distal portion (n = 3), and simultaneously in the gallbladder and the common duct other than distal portion (n = 2).

Abnormal pancreatic duct dilatation was noted in 4 of 43 cases (9.3%) of alcohol group and in 10 of 32 cases (31.3%) stone group ($P = 0.033$, Fisher's exact test). Bile duct dilatation was positive in 2 of 43 cases in 19 of 32 cases (59.4%) alcohol group, 59.4% (19/32) of stone group ($P = 0.000$, Fisher's exact test). Abnormal dilatation of either the pancreatic or bile duct had a statistical significance in differentiating between the two groups.

Infiltration scores of each abdominal compartment are summarized in Table 2. The overall degree of peripancreatic infiltration of right abdomen (sum of scores of RP + RSR + RIR) was almost same each other (2.40 ± 2.16) as in stone group (2.41 ± 2.12) ($P = 0.798$, likelihood ratio test). However, in the left abdominal compartments (LP + LSR + LIR), stone group (2.41 ± 1.79) showed a tendency of less peripancreatic infiltration than alcohol group, but not significant (3.67 ± 2.20) ($P = 0.153$). Among the six abdominal compartments, only the peritoneal aspect of left abdomen (or LP compartment) showed a significant difference in peripancreatic change between the two groups (1.67 ± 0.97 in alcohol group, 1.00 ± 0.92 in stone group, $P = 0.020$, likelihood ratio test).

The representative cases of acute pancreatitis caused by biliary stone and alcohol are presented in Figures 2 and 3, respectively.

DISCUSSION

Acute pancreatitis has numerous causes and an obscure pathogenesis. The exact pathogenetic mechanism of acute pancreatitis has not been completely established especially in the field of alcohol-induced pancreatic injury. The basic pathogenesis of acute pancreatitis is pancreatic autodigestion. Premature activation of zymogens within acinar cells, escape of activated enzymes from acinar cells and pancreatic ducts start the autodigestive process. It has not been established how alcohol abuse induces premature zymogen activation and release. However, the mechanism of gallstone pancreatitis is known via animal models. A stone impacted in the ampulla of Vater raises intraductal pressure. Increased pressure makes the pancreatic duct epithelium permeable to molecules of up to 25000 Da. Acute pancreatitis occurs when the pancreatic duct is perfused with active pancreatic enzymes, particularly when microvascular permeability is increased by the actions of histamine or prostaglandins. Thus, pancreatic zymogen activation and increased pancreatic duct permeability may

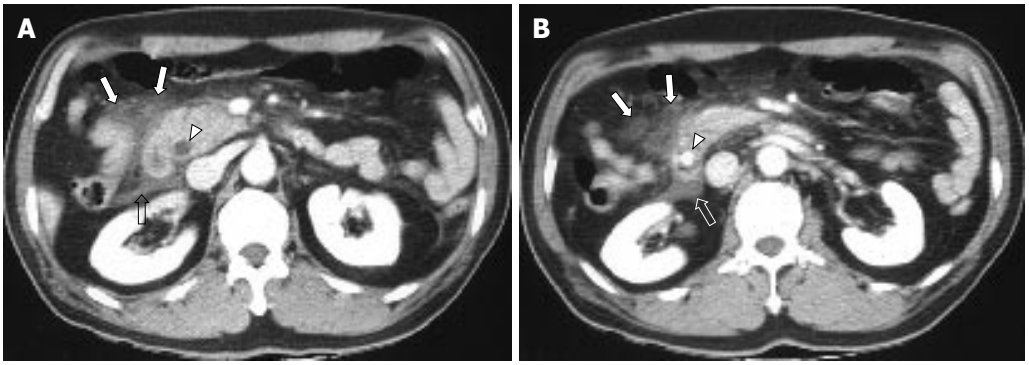

Figure 2 A 61-year old man with a clinical diagnosis of gallstone pancreatitis due to distal common bile duct stone. **A** and **B**: CT scans showing the peripancreatic infiltration or fluid collection predominantly in the right abdomen: score 1 infiltration in RP compartment (arrows), score 2 fluid collection in RSR compartment (open arrows). Distal common bile duct stone with bile duct dilatation also can be seen (arrowheads).

act sequentially initiating acute pancreatitis[1,4].

Despite this background knowledge of the pathogenesis of acute pancreatitis, we cannot readily explain why alcohol-induced pancreatitis forms more peripancreatic infiltration or fluid collection than gallstone pancreatitis, especially on the anterior aspect of the left-sided abdomen. As a candidate of the possible explanation for this phenomenon, we assume that the state of intoxication as well as the pain-killing effect of alcohol may play a role in masking acute symptoms of pancreatitis and make patients delay to be hospitalized. On the contrary, biliary stone itself often causes severe pain even before the initiation of acute pancreatitis. However, we cannot explain more prominent involvement of the anterior aspect of the left-sided abdomen by alcohol-induced pancreatitis. This tendency of a more aggressive form of pancreatitis by alcohol-induced disease than stone-associated disease is supported by some previous studies[5,6].

Most previous efforts to differentiate alcoholic and non-alcoholic pancreatitis have been based on laboratory data or clinical manifestations[7-10], while there are few studies using imaging modality. The authors of these studies have tried at best, to visualize a stone or bile sludge in the biliary system[11].

There was a prominent discrepancy between the rate of visible common bile duct stone on pre-contrast CT scan (15.6%) and the rate of common bile duct dilatation (59.4%) in our study. We think that this discrepancy is due to (1) a passed stone with post-inflammatory swelling of ampula of Vater, (2) a muddy stone in the common bile duct that could not be differentiated from bile on CT scan without measurement of Hounsfield unit, or (3) pancreatic swelling because of acute pancreatitis itself.

Only 15.6% of patients with gallstone pancreatitis showed calculi impacted in the ampulla of Vater although 68.8% were positive for gallstone pancreatitis if the entire biliary tree was included. In other words, 31.2% of gall stone pancreatitis patients did not show any calculi in the biliary system on abdominal CT scans. In most of these cases, diagnosis of gallstone pancreatitis was achieved through other imaging modalities such as ultrasonography or ERCP. Still in a small number of cases, serum bilirubin tests and/or patient history were needed for their clinical diagnosis. Similar situations are not uncommon in daily practice making ancillary CT findings meaningful in the clinical determination of acute pancreatitis etiology.

Based on our analysis, we assumed that the extent of the infiltration or fluid collections formed by acute pancreatitis could reflect the severity of the disease, as described by Balthazar *et al*[5]. Generally, infiltrations or fluid collections of acute pancreatitis tend to involve retroperitoneum rather than peritoneum because the pancreas is located in the retroperitoneal space[12]. On this point of view, we divided the retroperitoneum into superior and inferior compartments, and we also assumed that, initially, infiltrations might extend to retroperitoneum around pancreas (or superior compartment of retroperitoneum), then simultaneously further inferiorly along the retroperitoneal space and anteriorly to peritoneal space. Therefore, it is necessary to compartmentalize retroperitoneum into superior and inferior and score each compartment independently to weight on the significance of retroperitoneal space as an initially-preferred pathway of disease spread.

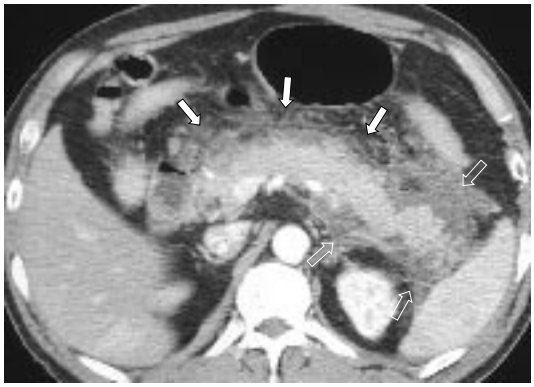

Figure 3 A 57-year old woman with score 1 infiltration in the RP and LP compartments (arrows) and score 2 fluid collections in the LP and LSR compartments (open arrows) is turned out to have a clinical diagnosis of alcoholic pancreatitis.

Population of our study was confined to the patients who had peripancreatic pathologic findings at initial abdominal CT. Therefore, results from our study cannot be applied to all patients with acute pancreatitis, but should be restricted to the moderate or severe cases in which CT shows peripancreatic pathology. However, we have met relatively severe forms of acute pancreatitis much more frequently in daily CT practice, and these are the real cases needing differentiation of their etiologies. So, we think the results from our study could be helpful.

Pancreatic necrosis is undoubtedly a very important factor for poor prognosis of acute pancreatitis. It has

been proven that patients with necrotizing pancreatitis have much a higher mortality and complication rate than patients with simple pancreatitis[13]. We regard a pancreatic necrosis as a mere fluid collection because we just want to know the meaning of the extent and distribution of fluid collection in differentiating major etiologies of acute pancreatitis. Whether the fluid is a necrotic pancreatic parenchyma or just a fluid remains unclear. However, we cannot agree that it is a potential limitation of our study.

Other limitations of our study are as follows, (1) Selection of patients was randomized but it was retrospective and not consecutive, possibly introducing potential selection bias; (2) The gold standard grouping of alcoholic and gallstone pancreatitis was the clinical diagnosis using medical records. Improper clinical history taking might play a role in incorrect determination of etiology of acute pancreatitis.

In conclusion, alcoholic pancreatitis tends to form more peripancreatic infiltrations or fluid collections than gallstone pancreatitis. This tendency is more prominent on the anterior (peritoneal) aspect of the left abdomen. Although clinical histories such as biliary colic or alcohol abuse and some CT findings favoring a gallstone pancreatitis usually are a major determinant of the etiology of acute pancreatitis, the degree and distribution of peripancreatic infiltration or fluid collection may have an ancillary role in differentiating the two major etiologies of acute pancreatitis especially in the case of insufficient clinical history or passed-out stone.

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CLINICAL RESEARCH

Characterization of pancreatic stem cells derived from adult human pancreas ducts by fluorescence activated cell sorting

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Abstract

AIM: To isolate putative pancreatic stem cells (PSCs) from human adult tissues of pancreas duct using serum-free, conditioned medium. The characterization of surface phenotype of these PSCs was analyzed by flow cytometry. The potential for pancreatic lineage and the capability of β-cell differentiation in these PSCs were evaluated as well.

METHODS: By using serum-free medium supplemented with essential growth factors, we attempted to isolate the putative PSCs which has been reported to express *nestin* and *pdx-1*. The Matrigel™ was employed to evaluate the differential capacity of isolated cells. Dithizone staining, insulin content/secretion measurement, and immunohistochemistry staining were used to monitor the differentiation. Fluorescence activated cell sorting (FACS) was used to detect the phenotypic markers of putative PSCs.

RESULTS: A monolayer of spindle-like cells was cultivated. The putative PSCs expressed *pdx-1* and *nestin.* They were also able to differentiate into insulin-, glucagon-, and somatostatin-positive cells. The spectrum of phenotypic markers in PSCs was investigated; a similarity was revealed when using human bone marrow-derived stem cells as the comparative experiment, such as CD29, CD44, CD49, CD50, CD51, CD62E, PDGFR-α, CD73 (SH2), CD81, CD105(SH3).

CONCLUSION: In this study, we successfully isolated PSCs from adult human pancreatic duct by using serum-free medium. These PSCs not only expressed *nestin* and *pdx-1* but also exhibited markers attributable to mesenchymal stem cells. Although work is needed to elucidate the role of these cells, the application of these PSCs might be therapeutic strategies for diabetes mellitus.

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Key words: Putative pancreas stem cell; *Nestin*; *pdx-1*; Phenotypic marker

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INTRODUCTION

Diabetes mellitus (DM)[1], one of the global diseases, is the basis for insulin deficiency either due to the inability of insulin secreting β-cells (type Ⅰ) or insulin resistance (type Ⅱ) in somatic cells. In the case of type Ⅰ DM treatment, islets of Langerhan transplantation has been demonstrated to restore normoglycemia[2]. Nevertheless, prevalent application is still limited by the shortage of donor pancreas, emphasizing the importance of producing β-cells *in vitro* before their transplantation into patients. The putative pancreatic stem cells (PSCs) have been reported in endocrine, acinar, and duct cells of human[3-5] and mouse studies[6-8], and the capacity to differentiate pancreatic lineage cells has been demonstrated *in vitro*. However, the existence and the biological role of putative PSCs in β-cell neo-regeneration is still doubtful[9].

Although the existence of PSCs has been reported in mice and humans, the methodological characterization of these PSCs is still ambiguous. The identification of PSC-specific biomarkers is required not only to define the PSCs operationally, but also to provide an efficient access for further purification. *Nestin*, an intermediate filament first identified in neuroepithelial stem cells, has been maintained as a marker of multi-lineage progenitor cells[10]. In regeneration studies of pancreas, some *nestin* positive cells have been observed[5]; nestin positive cells isolated from islets[11,12], mesenchymal cells[13], pancreatic ducts[14] and vascular endothelial cells[15] have been reported. In murine embryonic stem cell (mESC) studies, *nestin* positive cells could be selected and enriched by conventional medium cultivation for further neurogenesis[16], the application in pancreas was worth contemplating[17]. Pancreas duodenum homeobox-1 (*pdx-1*) is also essential for pancreatic development, insulin production, and glucose homeostasis. In animal models of partial pancreatectomy[18], diabetic models of streptozotocin (STZ) treatment in mice[19], injury and embryology studies[20,21], the expression of *pdx-1* was detected. For the biological role of the interaction with multiple transcription factors and co-regulators, it was thought as a direct indicator of cells with pancreatic differentiation potentials[22].

In the present study, we attempted to isolate putative PSCs from adult human pancreatic duct tissue rather than as in previous studies which used the animal model[13] or the human fetus[5]. Furthermore, to seek the potential biomarkers on these PSCs, the spectrum of phenotypic markers of human BMSCs was utilized and analyzed. These efforts attempt to investigate the properties of putative PSCs and demonstrate that β-cells could be induced by autogenous pancreatic tissue and possibly apply to diabetes therapy.

MATERIALS AND METHODS

Putative pancreatic stem cells (PSC) isolation

This research follows the tenets and regulations of the Declaration of Helsinki and has been reviewed by the Institutional Review Committee at Taipei Veterans General Hospital. Human pancreatic duct tissues at close proximity to the duct, originating from 4 identical donors, were dissected and digested by collagenase P (Roche Molecular Biochemicals, Mannheim, Germany) with HEPES-buffered saline for 7 h at 37℃. The digested tissue was washed two times with a HBS solution, pipetted up and down several times using a 10 mL syringe with a 22G needle, and placed into 10 cm Petri dishes with 10 mL of CMRL 1066 (5.6 mmol/L glucose, Gibco™, USA) media plus 10 mL/L Fetal bovine serum (FBS, Biological Industries, Israel). After two days incubation a sphere-like floating structure was observed. This suspended cell mass was collected by centrifugation, re-suspended using new serum-free ITSFn medium (composed: 1:1 of DMEM/F12, 0.6 g/L glucose, 25 μg/mL insulin, 100 μg/mL transferrin, 20 nmol/L progesterone, 60 μmol/L putrescine, 30 nmol/L selenium chloride, 2 mmol/L glutamine, 3 mmol/L sodium bicarbonate, 5 mmol/L HEPES buffer, 2 μg/mL heparin, 20 ng/mL human epidermal growth factor (EGF), 20 ng/mL human basic fibroblastic growth factor (b-FGF) and 20 ng/mL human hepatocyte growth factors, all growth factors were purchased from PerproTech, Israel) and placed into a new dish. The procedure was repeated twice to get rid of non-spherical masses and suspended cells, then the suspended cell mass was transferred to a 6 cm Falcon non-treated cultivation dish for plating, and cultivated using 10mL modified serum-free ITSFn medium. The medium was changed twice and sub-cultured once at a ratio of 1:5 in a week. The proliferation ability of putative PSC cells in passage 5, 10, 15, 20, 25 was examined by doubling time calculation.

Human bone marrow mesenchymal stem cell (BMMSC) isolation

Bone marrow aspirates were taken from the posterior iliac crest of normal adult donors (5 mL each; $n = 4$) and the isolation procedure followed our previous protocol[23]. Briefly, the bone marrow was washed twice with equal volume of PBS and centrifuged at 300 g for 10 min at room temperature. All washed cells were re-suspended in PBS to 10 mL and nucleated cells were isolated with a Percoll density gradient (diluted with equal volume of 1.073 g/mL Percoll solution, then centrifuged at 900 g for 30 min). The mononuclear cells (MNCs) were then suspended in plates. Expansion medium consisted of Dulbecco's modified Eagle's medium with 1 g/L glucose (DMEM-LG, Gibco) and 10% fetal bovine serum (FBS; Gibco) supplemented with 10 ng/mL bFGF, 10 ng/mL EGF, 10 ng/mL PDGF-BB (R&D), 100 Units/mL penicillin, and 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (Gibco). All of the nucleated cells were plated in 20 mL medium in a 75 cm^2 culture dish and incubated at 37℃ with 5 ml/L CO_2. After 24-48 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). These adherent cells were then cultured through 5 passages and used in flow cytometry studies.

RT-PCR detects the expression of nestin and pdx-1 in putative PSC

Trizol™ (Invitrogen, USA) reagent and GeneStrips™ (RNAture, USA) kits were employed for mRNA purification from putative PSC (passage 5). The Advantage RT-for-PCR Kit (Clontech; BD Biosciences, San Jose, CA) was used to synthesize the first strand of cDNA and 12 μL of extracted mRNA solution was utilized as the template. The experimental procedure followed the manufacturer's instructions. The sequence of primers used to detect human *nestin*, *pdx-1, insulin, glucagon* and *somatostatin* expression were as followed: *nestin*, forward: 5'-AGAGGGGAATTCCTGGAG-3', reverse: 5'-CTGAGGACCAGGACTCTCTA-3'; *pdx-1* forward: 5'-CCTTTCCCATGGATGAAGTC-3', reverse: 5'-TGTCCTCCTCCTTTTTCCAC-3'; *insulin* forward: 5'-CACACCTGGTGGAAGCTCTCT-3', reverse: 5'-GTAGAGGGAGCAGATGCTGGTA-3'; *glucagon* forward: 5'-ATCTGGACTCCAGGCGTGCC-3', reverse: 5'-AGCAATGAATTCCTTGGCAG-3'; *somatostatin* forward: 5'- TTCATCATCTACACGGC-3', reverse: 5'-GAGAG-

TAGAAGCAACCTACC-3'. Amplification was carried out with the program of 94℃ for 30 s to denature, 55℃ for 30 s for primer annealing and 72℃ for 30 s to elongate the PCR product for 30 cycles. The reaction was done on a total volume of 25 μL containing 0.5 μmol/L of each primer, 200 μmol/L dNTP, 2 units of Taq enzyme, and 5 μL of synthesized cDNA in reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.4, 1.5 mmol/L $MgCl_2$ and 100 μg/mL bovine serum albumin).

Pancreatic differentiation by basement-membrane-rich gel (Matrigel™)

The Matrigel™; a commercial preparation of murine basement membrane (BD biosciences, USA), was employed to induce pancreatic differentiation. Briefly, 1×10^4 cells of passage 10 and 20 were suspended with 1 mL medium and were placed on the top of the 6 cm plate coated with Matrigel™ (50 μL per cm^2) and were allowed to gel overnight before additional medium was added. Cell samples were taken for the following Dithizone and immunochemistry staining at different time points per week until the end of experimentation (4 wk).

Dithizone staining

Dithizone (DTZ, also named Diphenylthiocarbozone, Sigma, USA), which stains insulin-containing cells bright red, was used to quickly assess the presence of insulin-producing cells. The staining protocol followed was from the study by Shiroi, *et al* 2002[24].

Immunofluorescent staining

The sphere aggregated by PSC differentiated in Matrigel™ was dug and embedded by O.C.T. (Sakura Finetechnical Co., USA) for frozen section. Sections were fixed by ice-cold acetone (50 mL/L) for 2 min at 4℃, and blocked with 5 g/L skim milk at room temperature for 2 h. The sections were then incubated in rabbit anti-glucagon (1: 500, Abcam, ab11195), rabbit anti-glucagon (1:500, Abcam, ab 930), and mouse anti-insulin (1:100, BioGenex, MU029-UC) antibodies in 5 g/L skim milk at 37℃ for 2 h, washed twice by PBS, then followed with secondary antibody incubation (goat anti-mouse IgG with FITC conjugated for insulin detection, Jackson115-095-075, 1:500; goat anti-rabbit IgG with FITC conjugated for glucagon detection, Chemicon AP132F, 1:500; and goat anti- rabbit IgG- TRITC, Chemicon AP132R, 1:500). Specimens were washed in PBS three times after incubating with the secondary antibody and coverslips were applied using Fluoromount-G. The slide plating with HepG2 cells (ATCC) was used as a negative control (data not shown).

Measurement of insulin content/secretion

The differentiated cells from Matrigel™ were washed three times with PBS, and placed in 12-well dishes (Falcon, USA) with RPMI culture medium supplemented with 10 mL/L FBS and adjusted the glucose concentration up to 16 mmol/L (the RPMI contained 5 mmol/L glucose origin) then cultivated 48 h. The supernatant was collected and centrifuged to examine the insulin secretion, and kept at -80℃ before use. The attached cells were treated with cold acid-ethanol (0.1 N hydrochloric acid in absolute ethanol) and kept at 4℃ overnight to examine insulin content by ELISA (Mercodia, Sweden). The clear supernatants were used to investigate the intracellular insulin content and the values obtained were normalized relative to the total protein content (protein assay reagent, Bio-Rad, USA). The RIN-m5F insulinoma cell line (CRL-11605, ATCC) and undifferentiated PSCs were used as controls.

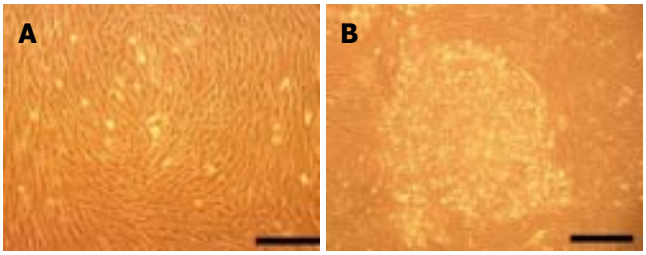

Figure 1 Putative PSC isolated from adult pancreas. **A**: Morphology of cultivated putative PSCs; **B**: Cells aggregate when saturated. bar = 100 μm.

Identification of cell phenotypic markers by FACS

Putative PSC of passage 10 and 20 was used for phenotypic marker identification by FACS. 1×10^5 cells were resuspended in 100 μL PBS and incubated with primary antibodies at 4℃ for 1 h with 1:100 dilutions. After washing twice with PBS, labeled cells were resuspended in 100 μL PBS with 1 μL goat anti-mouse IgG conjugated with FITC (Chemicon, AP124F) at 4℃ for 1 h, then examined by flow cytometry (BD, USA). The information of antibodies used in investigation was listed in Table 1.

RESULTS

Putative stem cells with nestin expression isolated from adult human pancreas

Putative PSCs with the property of sphere-like cell mass formation were cultivated by a series protocol of isolation and the divergent adhesion to bacteria Petri dishes. Cells with spindle-like shape were observed after plating and served with DMEM/F12 ITSFn serum free medium. The morphological homogeneity of putative PSC was demonstrated (Figure 1A). While the density of culture cells increased, the sphere-like cell aggregation was shown (Figure 1B). The expression of *nestin* and *pdx-1* were detected by RT-PCR, with sustained expression of both genes detected through 20 passages completing this study (RT-PCR examined, Figure 2). The protein level of nestin and pdx-1 was also examined by immunofluorescent staining (IF) revealing low protein intensity (data not shown). The experimental consistency was illustrated in the repeated examination of tissues from four individual donors.

The pancreatic differentiation potential of putative PSC

The differential competence of putative PSC was inspected by the growth in Matrigel™. The aggregation ability of the isolated PSCs (passage 10) was observed at d 3 after cell seeding (Figure 3A). Color formation was shown when stained with Dithizone at 4-wk after cell seeding, suggest-

Table 1 Comparative analysis of phenotype between human putative PSC and human BMMSC

Cell		Putative PSC								Human BMMSC	Information of antibodies
		Passage 10				Passage 20					
Antigen	Donor	1	2	3	4	1	2	3	4		
CD29		+	+	+	+	+	+	+	+	+	abcam, ab8238
CD44		+	+	+	+	+	+	+	+	+	abcam, ab6337
CD51		+	+	+	+	+	+	+	+	+	Ancell Corporation, USA
CD81		+	+	+	+	+	+	+	+	+	BD Pharmingen, clone JS81
SH2 (CD105)		+	+	+	+	+	+	+	+	+	ATCC, USA
SH3 (CD73)		+	+	+	+	+	+	+	+	+	ATCC, USA
CD14		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab760
CD38		-	-	-	-	-	-	-	-	+/-	abcam, ab1173
CD49b		+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD49d		+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD50		+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD54		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab1048
CD58		+	+	+	+	+	+	+	+	+/-	abcam, ab1420
CD61		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab7162
CD62E		+	+	+	+	+	+	+	+	+/-	abcam, ab6630
CD90		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab225
CD109		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	BD Pharmingen, 556039
EGFR		+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab30
PDGFR-alpha		+	+	+	+	+	+	+	+	+/-	R & D systems, USA
CD7		-	-	-	-	-	-	-	-	-	abcam, ab1249
CD34		-	-	-	-	-	-	-	-	-	abcam, ab8147
CD45		-	-	-	-	-	-	-	-	-	abcam, ab6329
CD62P		+	-	+	-	+	-	+	+	-	abcam, ab6632
CD62L		-	-	-	-	-	-	-	-	-	abcam, ab222
CD120a		-	-	-	-	-	-	-	-	-	Serotec, UK
AC133		-	-	-	-	-	-	-	-	-	Miltenyi Biotec., Germany

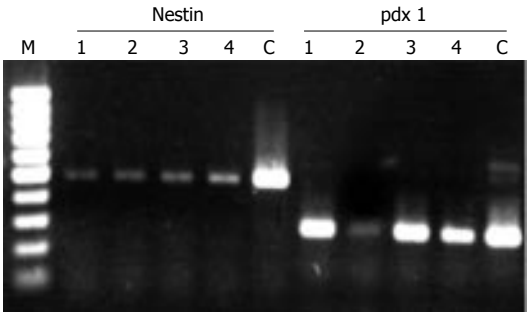

Figure 2 Nestin and pdx-1 expressed in putative PSCs after 5 passages of cultivation. cDNA oriented from 4 individual donors (lane No. 1 to 4), 100 bp marker (M) and the plasmid cloned human nestin and pdx-1 gene with positive control (C) were shown.

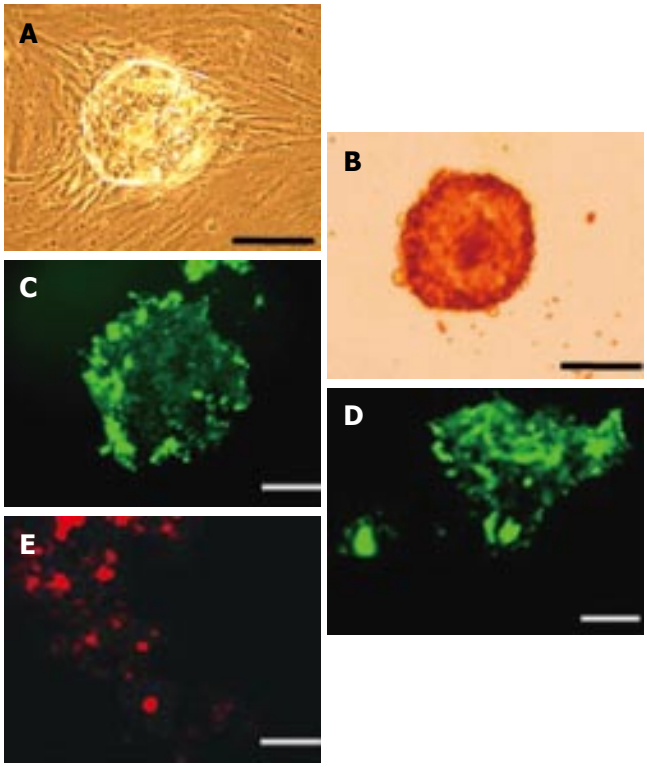

Figure 3 The differentiation of putative PSCs (4 wk). **A**: Growth in Matrigel™; **B**: Dithizone stain; **C-E**: Immunohistochemistry staining by anti-insulin (**C**), glucagon (**D**) and somatostatin (**E**) immunoglobulins. bar = 100 μm.

ing that cells went through β-cell differentiation in Matrigel™ (Figure 3B).

The expression of *insulin*, *glucagon* and *somatostatin* via RNA level was detectable in the putative PSCs (passage 10) growth in Matrigel™, comparatively; expression was barely illustrated in the group of cells without Matrigel™ (data not shown). Furthermore, the spheroid body of aggregated PSCs showed positive immune reactivity in IF staining (Figures 3 C-E). A similar result was observed in the examination of PSCs after 20 passages, suggesting the differentiation capacity of cells of pancreatic lineage could be preserved.

Measurement of insulin content in differentiated cells

The intracellular insulin content in the differentiating putative PSCs (the samples collected from individual donors were pooled together for measurement) were measured at

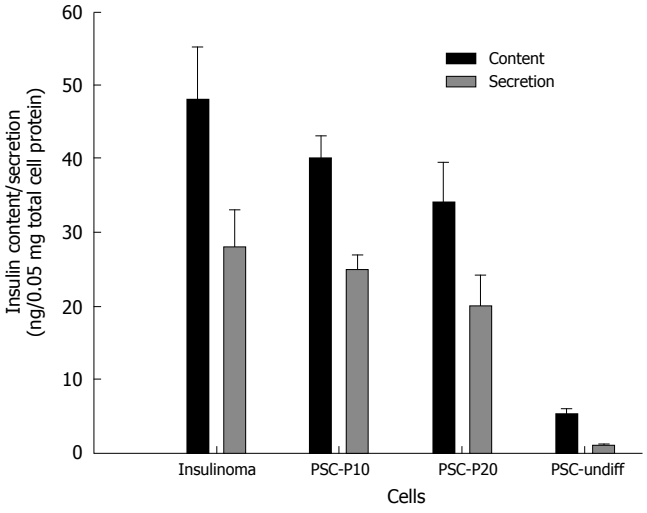

Figure 4 Measurement of Insulin content and secretion in differentiated putative PSCs.

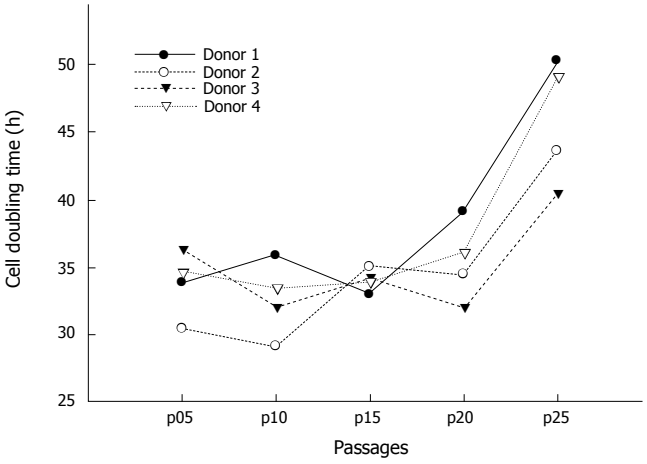

Figure 5 Cell doubling time of putative PSC.

4-wk after cell seeding on Matrigel™ (Figure 4). Compared to the undifferentiated putative PSCs, differentiated PSCs collected at passage 10 and 20 demonstrated the obvious increase in intracellular insulin content/secretion when normalized to the total protein content ($P > 0.01$).

Comparative phenotypic markers spectrum between putative PSC and BMMSC was detected by flow cytometry

To further investigate the potential phenotypic markers in PSCs, the biomarkers that are commonly examined in bone marrow- derived cells were used as the candidates for selection, and the human BMSC was employed as a comparative study (Table 1). The similar patterns of CD29, CD44, CD51, CD81, SH2 and SH3 were illustrated from both cells. Comparatively, differential intensity was demonstrated on the markers CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha; the stronger intensity was shown in putative PSCs. The markers of CD62P were revealed only in some PSC but not in BMMSC. No significant difference was shown between the putative PSC batches collected from 4 individual donors, moreover, the consistency was demonstrated in the studies of putative PSCs of passages 10 and 20.

Cell viability was monitored in long-period cultivation

A simplified cell proliferation method was used to monitor cell viability. The doubling time of 5, 10, 15, 20, 25 passages was illustrated in Figure 5. The cells proliferated rapidly at the beginning (32 h, approximately), however, a remarkable decrease was observed after 20 passages, and failure to proliferate occurred at approximately 30 passages.

DISCUSSION

The existence of putative PSC in pancreatic duct tissue of adult humans was illustrated in the present study. By using serum free medium with essential growth factors, cells with sphere-like mass aggregation were isolated. The putative PSC expressed both *nestin* and *pdx-1*, and these cells were able to differentiate into pancreatic lineage cells that express *insulin*, *glucagon* and *somatostatin* by Matrigel™. The potential biomarkers were evaluated by FACS, and the antibodies that identify common biomarkers of human bone marrow were used. As a comparative study of human BMSCs, the biomarkers of CD29, CD44, CD51, CD81, SH2 and SH3 (mesenchymal stem cell markers) were all detected on the surface of both cells and higher intensity in PSCs was illustrated in the markers of CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha. We also found that the expression of CD34, AC 133 (hematopoietic stem cell markers) and CD45 (endothelial marker) were not detected in either PSCs or BMSCs. The consistency of phenotypic patterns was demonstrated between individual (original cells from 4 identical donors) and the passages (to 20 passages).

Bone marrow (BM)-derived stem cells can be aspirated directly from donors. They can be cultured for *ex vivo* expansion. Previous studies demonstrated the pluripotency of these stem cells. They were able to differentiate into ectodermal[25,26], endodermal[26,27], mesodermal[27], hepatic[28], cardiac muscle[29] and skeletal muscle[30] progenitor cells. By using negative selection, a cell subpopulation isolated from bone marrow, muscle and brain cells have been shown to be able to differentiate into all three germ layers[31]. This type of mesenchymal stem cells, termed multipotent adult progenitor cells (MAPCs), have the remarkable potential to differentiate not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endodermal features at the single cell level. Recently, Zhang, *et al*[32] demonstrated that the *nestin*-positive progenitor cells isolated from human fetal pancreas, and these cells also have the phenotype markers identical to mesenchymal stem cells (MSCs). In agreement with these results, our results supported these findings and further isolation of *nestin*- and *pdx-1* -positive adult human pancreatic stem cells, which co-expressed the identical MSC markers. These interesting findings also provide evidence to support the interpretation of the study by Ianus *et al*[33], that bone marrow derived cells have the capacity to be competently pancreatic islet beta cells. Moreover, the differential capacities and properties of MSCs from different organs are

worth additional attention and may be investigated in a subsequent study.

Nestin is a well-discussed marker of the pancreatic stem cells in embryology, *in vitro* cultivation and pancreatectomy-regeneration in animal models[1,11,15,32]. However, a contradictory study provided evidence that *nestin*-lineage cells contribute to the microvasculature of pancreas but not endocrine cells of the islet[1]. The elegant experiment performed by Suzuki *et al*[3], had clearly demonstrated that the *nestin*-positive cells were isolated from the aggregated insulin-producing precursor and endothelium cells of pancreas by FACS assay[3]. This result provided a reasonable explanation that the *nestin* antigen is expressed in a much broader precursor population of pancreas, including differentiated cells. The *pdx-1*, compared to the *nestin*, is more specific for pancreatic differentiation[18-20]. In the present study, the strategy that both the *nestin*- and *pdx-1*- positive cells enriched by serum-free medium was employed followed the previous study of neuronal lineage stem cells isolated from murine embryonic stem cells[9,16]. Our data supported the claim that the *nestin*- and *pdx-1* expressed stem cells can be isolated from adult pancreatic ducts and possess the differentiation potential of the pancreatic lineage. Moreover, we found that the sustained expression of *nestin* and *pdx-1* in PSCs further exhibited and correlated to the stem cell characteristics of the insulin-producing ability, stable passage and long-term maintenance in vitro. Thus, *nestin* and *pdx-1* could not only be the markers of pancreatic stem cells but also play an important role in the self-renewal of beta progenitor cells.

In summary, we use the serum-free method and successfully isolate pancreatic stem cells from adult human pancreatic duct. These PSCs not only expressed the *nestin* and *pdx-1* but also exhibited the markers of mesenchymal stem cells. PSCs and the usage of serum free medium may avoid the potential immune problem of xenogenic protein contamination. Furthermore, this approach should overcome the ethical and immunologic concerns associated with the use of fetal tissues and embryonic stem cells. Although more work is needed to elucidate the role of these PSCs, the application of these PSCs can further be extended and used as an alternative source for therapeutic strategies of diabetes mellitus.

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CLINICAL RESEARCH

A population-based follow-up study on gallstone disease among type 2 diabetics in Kinmen, Taiwan

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Abstract

AIM: To assess the incidence of and risk factors for gallstone disease (GSD) among type 2 diabetics in Kinmen, Taiwan.

METHODS: A screening program for GSD was performed by two specialists who employed real-time abdominal ultrasound to examine the abdominal region after patients had fasted for at least eight hours. Screening, which was conducted in 2001, involved 848 patients diagnosed with type 2 diabetes. After exclusion of 63 subjects with prevalent GSD, 377 participants without GSD were invited in 2002 for a second round of screening. A total of 281 (74.5%) subjects were re-examined.

RESULTS: Among the 281 type 2 diabetics who had no GSD at the first screening, 10 had developed GSD by 2002. The incidence was 3.56% per year (95% CI: 1.78% per year-6.24% per year). Using a Cox regression model, age (RR = 1.07, 95% CI: 1.00-1.14), waist circumference (RR = 1.12, 95% CI: 1.01-1.29), and ALT (RR = 1.13, 95%CI: 1.01-1.26) appeared to be significantly correlated with development of GSD.

CONCLUSION: Older age is a known risk factor for the development of GSD. Our study shows that greater waist circumference and elevated ALT levels are also associated with the development of GSD among type 2 diabetics in Kinmen.

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Key words: Type 2 diabetes; Gallstone disease; Incidence density; Population-based study

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INTRODUCTION

Gallstone disease (GSD), a digestive disorder with multifactorial origins, is very common worldwide. Within the past few years ultrasonographic studies have provided estimates of GSD prevalence and of predisposing factors in various populations[1-5]. Although some controversy exists regarding the association between diabetes and GSD, population-based epidemiologic studies have demonstrated that diabetic subjects have an increased morbidity of GSD[6-8]. Moreover, our previous report showed that the prevalence of overall GSD among type 2 diabetics is higher than in other general Chinese populations when using the same methods for GSD assessment[5].

Previous study had explored the prevalence of GSD and associated factor among type 2 diabetics[5], and cross-sectional studies provided useful information of disease prevalence, however, they did not present the incidence or new cases in the study population. One must re-examine the population after a period of time in order to determine incidence and causal relationships between risk factors and

disease. From a preventive medicine viewpoint, primary prevention of GSD should focus on risk factors responsible for the occurrence of GSD. To explore the incidence of and risk factors for GSD is essential to prevent its development and the cholecystectomy caused by this complication, which is often insidious in nature. Therefore, it is necessary to conduct a population-based study which estimates GSD incidence. This is in part due to the fact that more than half of subjects with GSD are unaware of their condition and diagnosed cases seem to represent a selected group based on clinical studies[8]. Recently, however, a few population-based prospective studies have described the incidence and temporal relationship between the development of GSD and various risk factors among type 2 diabetics in Taiwan. The present study was conducted to explore the incidence and risk factors of GSD among type 2 diabetics in Kinmen, Taiwan based on a one-year follow-up period using real time abdominal ultrasound.

MATERIALS AND METHODS

Organization of gallstone disease screening for type 2 diabetics

Figure 1 shows the procedures for GSD screening between 2001 and 2002. Data used in this study were derived from a population-based screening for type 2 diabetes targeted to subjects aged 30 years or more in Kinmen, Taiwan, between January 1991 and December 1993. The details of the study design and execution have been described in full elsewhere[9]. The identification of type 2 diabetes was based on the WHO definition in 1985[10], namely, subjects with a fasting plasma glucose (FPG) $\geqslant$ 140 mg/dL or a 2 h postload glucose $\geqslant$ 200 mg/dL. Subjects with a history of type 2 diabetes and who had received medication were defined as known cases. However, in the GSD screening done in 2001, even patients who fulfilled the criteria of the revised WHO 1999 were enrolled. That is, additional patients with FPG $\geqslant$ 126 mg/dL and <140 mg/dL in 1991 to 1993 were also recruited[11]. A total of 1123 type 2 diabetics aged 30 and over were identified based on face-to-face interviews carried out by the Yang-Ming Crusade, a volunteer organization of well-trained medical students of National Yang-Ming University. After exclusion of those who migrated or died, the remaining 858 type 2 diabetics formed a cohort to receive first round abdominal ultrasound in 2001. A total of 440 (51.3%) subjects were examined in first screening for GSD. Sixty-three out of 440 type 2 diabetics were diagnosed with GSD. The overall prevalence of GSD was 14.4%, including single stone 8.0% (n = 35), multiple stones 3.2% (n = 14), and cholecystectomy 3.2% (n = 14) [5]. The 377 diabetics without GSD screened in 2001 were then invited by telephone calls or invitation letters in 2002 to receive a second round of abdominal examinations. Informed consent was obtained from all participants before the GSD screening[5].

Data collection and diagnosis of gallstone disease

In the present study, fasting blood samples were drawn by public health nurses. Overnight fasting serum and plasma samples (preserved with EDTA and NaF) were collected and kept frozen (-20℃) until analysis for measurements of biochemistry markers. FPG concentrations were determined using the hexokinase-glucose-6-phosphate dehydrogenase method with a glucose (HK) reagent ldt (Gilford, Cberlin, OH). The BMI, waist circumference, uric acid, and HbA_{1c} data were also collected during the GSD screening in 2001. The duration of type 2 diabetes in patients who were previously diagnosed with the disease was confirmed by the questionnaire. In addition, the screening protocol for GSD was performed in 2001 and 2002. GSD was diagnosed by two specialists using real-time abdominal ultrasound to examine the abdominal region after participants had fasted for at least 8 h. GSD was identified based on the presence of "movable hyperechoic material with acoustic shadow. Cases of GSD were classified as follows: single gallbladder stone, multiple gallbladder stones, and cholecystectomy, excluding gallbladder polyps. Cases were identified as any type of GSD among type 2 diabetics.

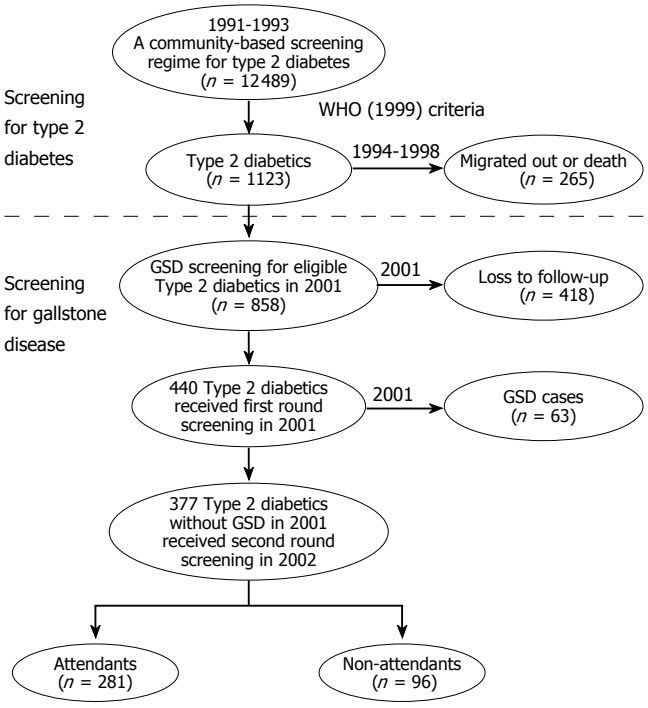

Figure 1 The procedure of screening for gallstone disease among type 2 diabetics during 2001-2002 in Kinmen.

Inter-observer reliability in ultrasound sonography

In order to set up a consistent diagnosis of GSD among two specialists, the Kappa statistic was used to assess inter-observer reliability among study specialists. A pilot study was performed with a randomly selected cohort (n = 50) of type 2 diabetics other than the study subjects. Our pilot study on inter-observer reliability showed a Kappa value for diagnosis of GSD of 0.77 (95%CI: 0.50-0.96).

Statistical analysis

Statistical analysis was performed using SAS software. The incidence of GSD was determined per year based on the ratio of the observed number of cases to the total number of patient-years at risk. Ninety-five percent confidence intervals (95% CI) for incidence were calculated using the Poisson distribution. In the univariate analysis, a t-test was

Table 1 Attendance rate of gallstone disease screening among type 2 diabetics in Kinmen

Variable	Eligible population	Screened population	Attendance rate
	n	*n*	(%)
Sex			
Male	167	121	72.5
Female	210	160	76.2
Age (yr)			
40-49	43	33	76.7
50-59	87	56	64.4
60-69	134	111	82.8
70+	113	81	71.7
Total	377	281	74.5

Table 2 Sex- and age-specific incidence of gallstone disease among type 2 diabetics in Kinmen

Variable	Incidence of any gallstone disease		
	No. of no GSD at first screening	New cases	Incidence density (% per year) (95%CI)
Sex			
Male	121	2	1.65 (0.03-5.10)
Female	160	8	5 (2.29-9.31)
P-value			0.12
Age (yr)			
40-49	33	0	0 (-)
50-59	56	2	3.57 (0.06-11.03)
60-69	111	4	3.6 (1.12-8.37)
70+	81	4	4.94 (1.53-11.47)
P-value			0.04
Total	281	10	3.56 (1.78-6.24)

Table 3 Univariate analysis of risk factors for the development of gallstone disease among type 2 diabetics in Kinmen

Variables	Development of gallstone disease			
	Yes	No	*P* value for *t* test	Definitions of disease condition
	Mean ± SD	Mean ± SD		
Age (yr)	69.90 ± 8.79	63.11 ± 10.72	0.04	-
Duration of diabetes (yr)	9.60 ± 0.84	9.44 ± 1.59	0.59	-
Fasting plasma glucose (mg/dL)	148.50 ± 18.45	144.94 ± 39.35	0.58	⩾ 126 mg/dL
HbA1c (%)	8.19 ± 0.93	8.33 ± 2.05	0.69	⩾ 7%
Systolic blood pressure (mmHg)	152.49 ± 13.91	145.08 ± 16.35	0.16	⩾ 140 mmHg
Diastolic blood pressure (mmHg)	89.55 ± 9.40	86.74 ± 9.52	0.36	⩾ 90 mmHg
Body mass index (Kg/m^2)	26.57 ± 1.07	25.36 ± 2.87	0.01	⩾ 27 kg/m^2
Waist circumference (cm)	89.74 ± 7.95	85.23 ± 7.52	0.000	⩾ 90 cm for males or ⩾ 80 cm for females
Total cholesterol (mg/dL)	220.09 ± 20.57	210.99 ± 26.82	0.000	⩾ 200 mg/dL
Triglyceride (mg/dL)	178.50 ± 38.95	144.28 ± 66.72	0.11	⩾ 200 mg/dL
AST (U/L)	25.40 ± 6.95	22.40 ± 8.15	0.01	⩾ 40 U/L
ALT (U/L)	31.36 ± 9.34	22.39 ± 10.28	0.01	⩾ 40 U/L
Uric acid (mg/dL)	6.07 ± 1.20	5.94 ± 1.29	0.76	⩾ 7 mg/dL for males or ⩾ 6 mg/dL for females

applied for continuous variables. Multiple Cox regression was used to investigate the independence of factors associated with development of GSD. $P < 0.05$ was considered statistically significant. The results are presented as mean ± SD.

RESULTS

Of 377 type 2 diabetics without GSD in 2001, 281 subjects attended the second round of abdominal ultrasound examinations in 2002. The overall attendance rate was thus about 74.5%. Subjects were considered as censored cases if the outcomes were not available. Table 1 shows that females had higher attendance rates than males (76.2% versus 72.5%), and old people (50-59 and over 70 year of age) had a slightly lower attendance rate than other age groups.

Table 2 presents gender-specific and age-specific one-year incidences of GSD. Overall incidence of GSD was 3.56% per year (95%CI: 1.78% per year-6.24% per year). This incidence shows a clear trend with age ($P = 0.04$) with values increasing monotonically: from 0% per year at age 40-49 years to 3.57% per year at age 50-59 years, 3.60% per year at age 60-69 years to 4.94% per year at older ages. There was no consistent pattern by age group. Females had a slightly higher incidence (5.00% per year versus 1.65% per year, $P = 0.12$) than males, although the gender difference was not statistically significant.

Table 3 shows the risk factors for the development of GSD in type 2 diabetics by univariate analysis. The risk factors that were significantly related to the development of GSD included age (*t*, T = 1.98, $P = 0.04$), BMI (T = 3.18, $P = 0.01$), waist circumference (T = 8.16, $P = 0.0001$), total cholesterol (T = 4.94, $P = 0.0001$), AST (T = 2.83, $P = 0.01$), and ALT (T = 2.69, $P = 0.01$).

To assess the independence of the contributions of these factors to the development of GSD, the significant variables from univariate analysis for GSD were further examined using a Cox regression model including age, BMI, waist circumference, total cholesterol, AST, and ALT. As Table 4 shows, age (RR = 1.07, 95%CI: 1.00-1.14), waist circumference (RR = 1.12, 95%CI: 1.01-1.29), and ALT (RR = 1.13, 95%CI: 1.01-1.26) appeared to be independently correlated with development of GSD.

DISCUSSION

Incidence and risk factors for the development of gallstone disease

Abdominal ultrasound for GSD screening is viewed as a robust method. Previous clinical studies have shown reliable positive (0.99-1.00) and negative (0.90-0.96) predictive

Table 4 Cox regression model of risk factors associated with the development of gallstone disease among type 2 diabetics in Kinmen

Variables	Development of gallstone disease (yes *vs* no)	
	Relative risk	(95% CI)
Sex (female *vs* male)	2.60	0.52-13.11
Age (yr)	1.07	1.00-1.14
Body mass index (Kg/m^2)	1.03	0.57-1.34
Waist circumference (cm)	1.12	1.01-1.29
Total cholesterol (mg/dL)	1.01	0.98-1.03
AST (U/L)	0.91	0.77-1.07
ALT (U/L)	1.13	1.01-1.26

values of ultrasonographic diagnosis[12]. In the present study, the annual incidence of overall GSD was higher than that in other general population-based studies[12-15], implying that type 2 diabetes might be a positive risk factor for GSD development. Possible pathogenic reasons are that type 2 diabetes combined with GSD might induce acute cholecystitis more often and have a higher probability of progression to septicemia than does gallbladder dysfunction in non-diabetic patients[16], and late-onset diabetic patients have a higher lithogenic bile index than non-diabetics after adjustment for sex and age[17]. In addition, hyperglycemia in diabetic subjects might exert effects on gallbladder motility[18].

An association between GSD and use of exogenous estrogens was confirmed[19]. The lithogenic effects of estrogen are mediated in part by an increase in bile cholesterol saturation[19]. However, previous studies showed that cholesterol GSD is common in Western populations whereas pigment GSD is major components in Taiwan[1]. Unlike result for cholesterol GSD, our results did not show a causal relationship between female sex and development of pigment GSD. The different findings for Orientals from those shown for Occidentals suggest that cholesterol GSD has not yet become a major GSD component in Taiwanese diabetic populations.

Using both univariate analysis and a multiple Cox regression model, our study also demonstrated that age is a significant risk factor for the development of GSD. This result is not concordant with results from other studies that had longer screening intervals[8,13,15]. Larger amounts of cholesterol secreted by the liver and decreases in the catabolism of cholesterol to bile acid were observed in the elderly[20]. Although the long-term exposure to many other risk factors in the elderly might account for their increased chance of developing GSD[5], age still remained a major factor leading to GSD development, irrespective of locality, standard of living, or after adjustment for other demographic and clinical characteristics in the multivariate analysis.

Several population-based studies demonstrated that liver cirrhosis represents a strong risk factor for GSD[21,22]. The annual incidence of GSD in patients with cirrhosis appears to be about eight times higher than in the general population[21]. Alanine aminotransferase (ALT) has, for some time, been viewed as a sensitive indicator of liver-cell injury[23]. Currently, the determination of serum ALT levels constitutes the most-frequently applied test for the identification of patients suffering from liver disease. This parameter also acts as a surrogate marker for disease severity and/or as an index of hepatic activity[24]. Our results showing that elevated ALT levels constitute higher risk of GSD development suggest that appropriate integrated diagnosis and therapy in the early stage of liver dysfunction might eventually enable us to prevent incident GSD. Instead of being a sign of more serious liver disease like liver cirrhosis, further studies should be conducted to explore the possibility of whether elevated ALT levels is an indicator for GSD because it indicates a fatty liver (and thus a high BMI), and early stage of chronic liver disease.

Obesity could raise the saturation of bile by increasing biliary secretion of cholesterol, the latter probably depending on a higher synthesis of cholesterol in obese subjects[25]. Being overweight at baseline was strongly associated with the incidence of GSD which was also suggested in epidemiologic studies[13]. In this follow-up study, we found that a higher waist circumference rather than a higher BMI was significantly and positively associated with GSD development. Thus abdominal obesity might be more important than BMI for identifying diabetics at high risk of GSD. One possible reason is that due to a high correlation between BMI and waist circumference (r = 0.64), waist circumference might explain the effect of BMI on GSD development in type 2 diabetic subjects. Another possible reason is that a large waist circumference might be an unambiguous indicator of excess body fat, except in the presence of abdominal tumors or ascites, and might be a better estimate of overall body fat than is BMI. In addition, from the biological perspective, BMI becomes primarily a surrogate of lean body mass when BMI and height-adjusted waist circumference are included in the same model, because the variation in BMI attributable to adiposity is essentially controlled by the height-adjusted waist circumference variable[14]. Nevertheless, further epidemiological and etiologic investigations are needed to explore the pathophysiological mechanism underlying gender-related differences in waist circumference and GSD among diabetics.

Methodological considerations

Although using a follow-up study design can clarify the temporal relationship of potential risk factors and the development of GSD, there are some limitations in the present study. First, the characteristics pertinent to the risk of type 2 diabetes for study subjects were not significantly different from non-respondents (except for age), indicating that subjects who did not return for follow-up might have more severe GSD. Also, we assumed that all the new GSD cases occurred in 2002. Since additional GSD cases could occur in subsequent years, the incidence of GSD may be underestimated. Second, all the patients had diabetes in the study population, therefore, an evaluation of the extent of GSD incidence in subjects without diabetes was difficult. Third, we did not attempt to estimate the incidence

of gallstone formation but rather the incidence of newly screened GSD. Our analysis only focused on clinically relevant GSD. Fourth, due to a shorter follow-up period, we did not have a large enough sample size to estimate the "true" effects between potential risk factors and the incidence of GSD. Further long-term studies should be conducted to explore the morbidity of GSD and plausible biological mechanisms underlying its development.

In conclusion, our reports show that the incidence of GSD is 3.56% per year. Significant risk factors for the development of GSD include not only older age, but also, higher waist circumference and elevated ALT levels among type 2 diabetics.

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RAPID COMMUNICATION

Decrease of serum carnitine levels in patients with or without gastrointestinal cancer cachexia

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Abstract

AIM: To evaluate the levels of serum carnitine in patients with cancer in digestive organs and to compare them with other cancers in order to provide new insights into the mechanisms of cachexia.

METHODS: Fifty-five cachectic patients with or without gastrointestinal cancer were enrolled in the present study. They underwent routine laboratory investigations, including examination of the levels of various forms of carnitine present in serum (i.e., long-chain acylcarnitine, short-chain acylcarnitine, free carnitine, and total carnitine). These values were compared with those found in 60 cancer patients in good nutritional status as well as with those of 30 healthy control subjects.

RESULTS: When the cachectic patients with gastrointestinal cancer were compared with the cachectic patients without gastrointestinal cancer, the difference was -6.8 μmol/L in free carnitine ($P < 0.005$), 0.04 μmol/L in long chain acylcarnitine ($P < 0.05$), 8.7 μmol/L in total carnitine ($P < 0.001$). In the cachectic patients with or without gastrointestinal cancer, the difference was 12.2 μmol/L in free carnitine ($P < 0.001$), 4.60 μmol/L in short chain acylcarnitine ($P < 0.001$), and 0.60 μmol /L in long-chain acylcarnitine ($P < 0.005$) and 17.4 μmol/L in total carnitine ($P < 0.001$). In the cachectic patients with gastrointestinal cancer and the healthy control subjects, the difference was 15.5 μmol/L in free carnitine ($P < 0.001$), 5.2 μmol /L in short-chain acylcarnitine ($P < 0.001$), 1.0 μmol/L in long chain acylcarnitine ($P < 0.001$), and 21.8 μmol/L in total carnitine ($P < 0.001$).

CONCLUSION: Low serum levels of carnitine in terminal neoplastic patients are decreased greatly due to the decreased dietary intake and impaired endogenous synthesis of this substance. These low serum carnitine levels also contribute to the progression of cachexia in cancer patients.

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Key words: Acetyl carnitine; Nutritional status; Anorexia; Malnutrition; Fatigue

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INTRODUCTION

Cancer cachexia is a condition characterized by anorexia, chronic nausea, early satiety, muscle wasting, involuntary weight loss, lipolysis, weakness, fatigue, impaired mental and physical performance, decreased capacity of wound healing, impaired immunological function, and compromized quality of life. Cancer cachexia is associated with poor survival and decreased tolerance to both radiotherapy and chemotherapy[1].

The prevalence of cachexia is about 60% in terminal cancer patients[2]. Anorexia and nausea exacerbate the detrimental effects of tumor-related changes in protein metabolism on nutritional status, leading to increased morbidity and mortality[3].

The mechanisms responsible for cachexia in cancer patients are poorly understood. One of the factors contributing to weight loss is the reduced food intake, which may be caused by a decrease in appetite or a tumor treatment[4], mechanical obstruction of the gastrointestinal (GI) tract or intestinal malabsorption.

A decrease in food intake, combined with a decrease in physical exercise, leads to a decline in muscle mass and power.

Several studies have suggested that metabolic control of food intake also exists, in which the biochemical partitioning between fatty acid oxidation and synthesis is a vital signal indicating catabolic or anabolic energy status[5]. Changes in energy metabolism influence energy intake via energy signals.

Energy signals are independent from body mass, but they inform the brain on the metabolic switch occurring subcellularly between fatty acid oxidation and synthesis[6]. The combination of reduced energy intake and increased energy expenditure in cancer patients results in substantial weight loss. It has been shown that weight loss in cancer patients comprises both muscle mass and fat mass.

When there is a cancer, several metabolic changes are present in the whole body. It could be possible that one of these factors is represented by a decrease of endogenous synthesis of carnitine or by a reduction of exogenous assumption of carnitine.

Carnitine is a naturally occurring substance required for energy metabolism. In omnivores approximately 75% of carnitine sources come from diet and 25% from endogenous synthesis[7]. Under normal conditions, omnivores absorb about 70%-80% of dietary carnitine[8].

Human skeletal muscle, heart, liver, kidney and brain are capable of biosynthesizing carnitine from motioning and lysine to its immediate precursor gamma butyrobetaine[9].

Carnitine is essential for the transport of long chain fatty acids across the mitochondrial membrane for fatty degradation and energy production and has the ability to shuttle short chain fatty acids from inside the mitochondria to the cytosol.

In a previous study[10], we observed low serum levels of carnitine in patients with tumoral cachexia, which may be due to a decreased availability of carnitine in the diet or to the altered endogenous biosynthesis.

The aim of the present study was to evaluate the levels of serum carnitine in patients with cancer in digestive organs and to compare them with other cancers in order to provide new insights into the mechanisms of cachexia.

MATERIALS AND METHODS

Fifty-five patients eligible for this study had advanced malignancies localized in various parts of the body. The patients were in the terminal phase of their disease and only palliation was requested. All of them showed a weight loss above 5% in the 6 mo prior to enrollment in the study. Thirty cachectic patients with gastrointestinal cancer (16 males and 14 females, mean age 47.4 ± 7.6 years) had the following diagnoses: cancer of the stomach in 6, cancer of the small bowel in 6, cancer of the colon in 10, cancer of the rectum in 8. Twenty cachectic patients without gastrointestinal cancer (13 males and 12 females, mean age 49.1 ± 6.4 years) had the following diagnoses: lung cancer in 5, renal cell carcinoma in 5, malignant melanoma in 5, bladder cancer in 5, prostate cancer in 2, and breast cancer in 3. Forty of them underwent surgical intervention, while 31 received one or more chemotherapeutic treatments and 6 received radiotherapy. We also enrolled two groups of control subjects.

Sixty cancer patients were in good nutritional status (25 males and 35 females, mean age 48.6 ± 8.4 years). Their diagnoses were as follows: colorectal carcinoma in 10, lung cancer in 15, breast cancer in 10, gastric cancer in 3, bladder cancer in 7, renal cell carcinoma in 4, prostate cancer in 10, and testicular cancer in 1.

Table 1 Characteristics of the study population (mean ± SD)

Parameter	Cachectic patients with gastrointestinal cancer (30)	Cachectic patients without gastrointestinal cancer (25)	Non cachectic patients with cancer (60)	Healthy subjects (30)
Sex M/F	16/14	13/12	25/35	15/15
Mean age (yr)	47.4 ± 7.6	49.1 ± 6.4	48.6 ± 8.4	51.3 ± 5.2
Heigth (cm)	158.9 ± 6.8	160.6 ± 7.1	159.4 ± 7.8	160.2 ± 6.4
Weigth (kg)	46.8 ± 7.6	51.4 ± 6.9	60.2 ± 7.4	63.8 ± 11.3
SAP (mmHg)	151.8 ± 12.4	156 ± 13.2	152.4 ± 14.7	150.3 ± 16.8
DAP (mmHg)	84.2 ± 8.7	86.4 ± 7.1	80.1 ± 9.7	80.2 ± 10.6
Heat Rate bpm	94.2 ± 9.8	86.2 ± 11.1	84.2 ± 7.1	81.2 ± 10.2

A second control group consisted of 30 healthy individuals aged 51.3 ± 5.2 years. The patient group (n = 4) was examined in the morning between 8:00 a.m. and 10:00 a.m. after an overnight fast. Then, venous blood samples were taken and stored in tubes containing ethylenediamine tetra acetic acid (EDTA) or heparin (Table 1).

Serum or plasma was obtained by centrifugation. Serum was analyzed immediately, while plasma and urine were stored at -20℃ before analysis. Serum carnitine levels were determined using the Cederblad and Lindstedt method modified by Brass and Hoppel[7]. Plasma was treated with perchloric acid (final concentration 3% vol: vol) and centrifuged for 2 min at 10000 r/min. Long-chain acylcarnitine was extracted from the pellet after alkaline hydrolysis, while free and short-chain acylcarnitines were extracted from the supernatant. The sum of short-chain acylcarnitine, free carnitine, and long-chain acylcarnitine was considered the serum of total carnitine level.

Creatinine concentrations were determined using a kinetic colorimetric reaction in the same samples used to measure the carnitine concentrations. All laboratory tests were performed using standard laboratory procedures.

All subjects followed a daily diet consisting of 1800 kcal/d with a content of total cholesterol < 300 mg/d, 50% carbohydrates, 20% proteins, and 30% fats (of which 10% were saturated fatty acids, 10% unsaturated fatty acids, and 10% polyunsaturated fatty acids).

Statistical analysis

The results are presented as mean ± SD. The following two-tailed tests at $P < 0.05$ were used in the study: the Mann-Whitney U-test in the case of two independent samples and the Spearman's rank correlation coefficient test for univariate relationships between variables. In order to evaluate the independent effects of covariates on carnitine concentration, a stepwise multiple linear regression analysis was performed.

Data were analyzed using the statistical package SPSS for Windows 7.5 (SPSS *Inc.*, Chicago, IL, USA).

RESULTS

Comparison of baseline characteristics

The four groups of subjects were comparable in age, height, systolic and diastolic pressure. The difference in weight was 4.50 kg ($P < 0.05$) between cachectic

patients with gastrointestinal cancer and cachectic patients without gastrointestinal cancer, 13.40 kg (P < 0.001) between cachectic patients with gastrointestinal cancer and neoplastic non-cachectic patients, and 17.0 kg between cachectic patients with gastrointestinal cancer and healthy subjects (P < 001). The difference in weight was 8.80 kg (P < 0.001) between cachectic patients without gastrointestinal cancer and non-cachectic patients, and 12.40 kg (P < 0.001) between cachectic patients without gastrointestinal cancer and healthy subjects.

Comparison of laboratory parameters

When the cachectic patients with gastro-intestinal cancer were compared to the cachectic patients without gastrointestinal cancer, the difference was 12.5 mg/dL in triglycerides (P < 0.001), 26.7 IU/L in ALP (P < 0.001) and 802.6 ng/mL in CEA (P < 0.001).

The difference was 6.8 μmol/L in free carnitine (P < 0.005), 0.04 μmol/L in long chain acylcarnitine (P < 0.05), 8.7 μmol/L in total carnitine (P < 0.001).

When the cachectic patients with gastrointestinal cancer were compared to non cachectic patients with cancer, the difference was 5.8 mg/dL in bun (P < 0.001), 7.00 mg/dL in glucose (P < 0.001), 34.1 mg/dL in total cholesterol (P < 0.001), 31.3 mg/dL in triglycerides (P < 0.001), 8.60 IU/L in AST (P < 0.001), 45.8 IU/L in ALP (P < 0.001) and 815.6 ng/mL in CEA (P < 0.001). The difference was 12.2 μmol/L in free carnitine (P < 0.001), 4.60 μmol/L in short-chain acylcarnitine (P < 0.001), and 0.60 μmol/L in long-chain acylcarnitine (P < 0.005) and 17.4 μmol/L in total carnitine (P < 0.001).

When the cachectic patients with gastrointestinal cancer were compared to the healthy control subjects, the difference was 27.60 mg/dL in total cholesterol (P < 0.001), 25.8 mg/ dL in triglycerides (P < 0.001), 13.0 IU/L in ALT (P < 0.001), 15.60 IU/L in AST (P <0.001), and 76.8 IU/L in ALP (P < 0.001) and 845.1 ng/mL in CEA (P < 0.001). The difference was 15.5 μmol/L in free carnitine (P < 0.001), 5.2 μmol/L in short-chain acylcarnitine (P < 0.001), 1.0 μmol/L in long-chain acylcarnitine (P < 0.001), and 21.8 μmol/L in total carnitine (P < 0.001).

When the cachectic patients without gastrointestinal cancer were compared to the non cachectic patients with cancer, the difference was 3.2 mg/dL in bun (P < 0.01), 7.5 mg/dL in glucose (P < 0.001), 27.3 mg/dL in total cholesterol (P < 0.001), 18.8 mg in triglycerides (P < 0.001), 5.9 IU/L in AST (P < 0.01), 19.10 in ALP (P < 0.001), 5.40 μmol/L in free carnitine (P < 0.01), 3.10 μmol/L in short-chain acylcarnitine (P < 0.005), 8.7 μmol/L in total carnitine (P < 0.001).

When the cachectic patients without gastrointestinal cancer were compared to the healthy controls, the difference was 5.40 mg/dL in Bun (P < 0.005), 20.8 mg/dL in total cholesterol (P < 0.001), 13.3 mg/dL in triglycerides (P < 0.001), 8.7 IU/L in ALT (P < 0.001), 12.9 IU/L in AST (P < 0.001), 50.1 IU/L in ALP (P < 0.001), 8.7 μmol/L in free carnitine (P < 0.001), 3.7 μmol/L in short-chain acylcarnitine (P < 0.01), 0.6 μmol/L in long-chain acylcarnitine L (P < 0.005), 13.1 μmol/L in total carnitine (P < 0.001).

Table 2 Laboratory parameters of subjects included in our study (mean ± SD)

Parameter	Group 1 (30 pts)	Group 2 (25 pts)	Group 3 (60 pts)	Group 4 (30 pts)
Bun (mg/dL)	35.6 ± 5.4	38.2 ± 5.1	41.4 ± 4.7	32.8 ± 7.9
Glucose (mg/dL)	68.4 ± 9.1	67.9 ± 8.7	75.4 ± 9.1	71.2 ± 9.4
Creatinine (mg/dL)	0.87 ± 0.37	0.91 ± 0.25	1.06 ± 0.20	0.97 ± 0.21
Total Cholesterol (mg/dL)	160.1 ± 13.9	166.9 ± 14.2	194.2 ± 8.7	187.7 ± 10.8
Triglycerides (mg/dL)	144.3 ± 13.2	156.8 ± 10.9	175.6 ± 9.6	170.1 ± 9.8
ALT (IU/L)	49.7 ± 9.8	45.4 ± 7.9	46.1 ± 7.8	36.7 ± 4.4
AST (IU/L)	51.4 ± 8.9	48.7 ± 7.4	42.8 ± 9.6	35.8 ± 6.7
ALP (IU/L)	241.8 ± 22.6	215.1 ± 20.7	196 ± 16.4	165 ± 18.2
CEA (ng/mL)	847.2 ± 196.4	44.6 ± 13.1	31.6 ± 15.8	2.1 ± 1.4

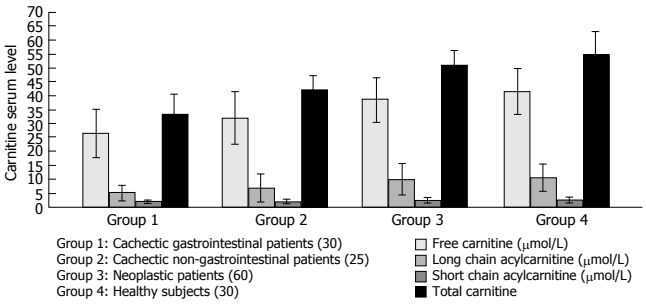

Figure 1 Comparison of carnitine serum levels in four subject groups.

When non-cachectic cancer patients were compared to the healthy controls, the difference was 8.6 mg/dL in BUN (P < 0.001), 4.2 mg/dL in glucose (P < 0.05), 6.5 mg/dL in total cholesterol (P < 0.005), 5.5 mg in triglycerides (P < 0.01), 9.4 IU/L in ALT (P < 0.001), 7.00 IU/L in AST (P < 0.001), and 31.01 IU/L in ALP (P < 0.001).

In comparison of serum plasma carnitine in controls and neoplastic patients, there were no significant differences (Table 2).

The correlation studies in patients with gastrointestinal cancer showed a strong correlation between the following parameters: total carnitine (r = 0.75), short-chain acyl-carnitine (r = 0.70), long-chain acyl-carnitine (r = 0.78), free carnitine (R = 0.70), weight loss and total carnitine (R = 0.81), short chain acylcarnitine (r = 0.78), long chain acylcarnitine (r = 0.71), free carnitine (r = 0.74) and CEA.

The correlation studies in patients also showed a correlation between total carnitine (r = 0.61), short chain acylcarnitine (r = 0.65), and long chain acylcarnitine (r = 0.64) in free carnitine (r = 0.61) and weight loss.

No correlation was found between total carnitine or the fractions of carnitine and other biohumoral and demographic characteristics (Figure 1).

DISCUSSION

The current study demonstrated that patients with gastrointestinal cancer cachexia showed a significant decrease in fractions and total carnitine serum levels in

comparison with controls.

Malignant lesions are generally associated with weight loss, due to delay in the establishment of a diagnosis. Gastrointestinal cancer is characterized by malabsorption with excess fecal loss of ions and water, in addition to nutrient. Cachexia is associated with inflammatory or neoplastic condition that evokes an acute-phase response with an increase of cytokine production (such tumor necrosis factor, interleukin 1, 6 and interferon), and feeding does not reverse the macronutrient changes.

Patients with gastrointestinal cancer may also experience anorexia secondary to food adversion.

Gastrointestinal cancer presents with symptoms and signs such as loss of appetite, abdominal discomfort, decreased gastrointestinal transit with obstructive symptoms, weight loss, weakness, nausea and vomiting.

Diversion to food can be attributed to the location of the tumor, its rate of growth and size[11].

Patients with growing tumors and poor intake due to bowel obstruction or severe dysphagia present with progressive nutritional deprivation as the main mechanism of cachexia.

The low plasma carnitine concentration in these patients is probably a consequence of decreased intake of carnitine and the carnitine precursors L-lysine and L-methionine.

Carnitine is critical for normal skeletal and heart muscle bioenergetics.

The decrease of carnitine and its derivates in patients with cachexia can explain the sarcopenia.

In fact, skeletal muscle is the main reservoir in the body and possesses a carnitine concentration at least 50 to 200 times higher than in blood, where the average concentration is about 50 μmol/L[12].

Administration of exogenous L- carnitine might improve the nutritional status in patients without gastrointestinal cancer and enhance muscle mass exerting a favorable effect on chronic fatigue syndrome in cancer patients.

The values of serum carnitine determination are questionable in respect to consequences and interpretation. This is due to the fact that serum carnitine represents approximately 3% of total body carnitine.

Rates of changes beyond the limits of 2%-3% in 1 mo can be considered abnormal. However, rates of changes within these limits may also be abnormal if divergent changes are seen in different body compartments (for example, depletion of skeletal muscle plus fluid overlood caused by cardiac, hepatic, or renal disease; hypoalbuminemia or intravenous hydration)[13,14].

Carnitine is required for long-chain fatty acid oxidation and assists in removing accumulated acyl groups from the mitochondria and plays an important role in detoxification[15]. The reduced levels of carnitine seem to be related with both malabsorption and impaired carnitine production. A decrease in serum carnitine explains not only the sarcopenia but also indicates a bioenergetic deficit with the physical and mental fatigue detectable in patients[16].

An increase in resting energy expenditure may contribute to weight loss in cancer patients. L-carnitine has been shown to have physiologic effects on metabolism in cachexia models, presumably because of its ability to increase fatty oxidation[17].

The decrease in total serum carnitine is associated with a decrease in acylcarnitine, a fundamental substance in brain metabolism. Deficiency in acyl carnitine can induce behavioral and cognitive changes (anxiety, depression and malignancy or treatment-related anorexia)[18,19].

The direct relationship between total serum carnitine and short-chain and long-chain acylcarnitine levels in patients with gastrointestinal cancer cachexia and CEA suggests that the observed decrease in serum carnitine levels is directly influenced by the activity of gastrointestinal cancer.

In fact, malnutrition triggers a vicious circle in which neoplastic patients produce additional amounts of cytokines. The decrease in total and fractions of carnitine in patients without gastrointestinal cancer cachexia in comparison with cancer patients in good nutritional status and healthy control subjects suggests the alteration of endogenous biosynthesis of carnitine due to elevated cytokine production associated with cachexia. In fact, cachexia has a multifactorial pathogenesis and involves several neuronal systems that modulate production and transport of cell energy, such as hormones, neuropeptides, cytokines and neurotransmitters (serotonin and dopamine)[20].

Since therapeutic options currently available for the treatment of these patients are not successful,

administration of carnitine may allow us to correct the unregulated immune response and improve the total energy expenditure[21]. The total energy expenditure involves in fact resting energy expenditure (approximately 70%), voluntary energy expenditure (25%), and energy expenditure in digestion (5%).

Therefore, there is no evidence that supports the use of nutritional or pharmacological intervention to improve the likelihood of either survival or improved anti-neoplastic interventions[22,23]. L-carnitine and L-acetylcarnitine may be effective at limiting the demands placed on cachectic patients by acute stresses, such as sudden increases in physical activity, immunological challenge or acute and chronic malnutrition.

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RAPID COMMUNICATION

Conserved balance of hepatocyte nuclear DNA content in mononuclear and binuclear hepatocyte populations during the course of chronic viral hepatitis

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Abstract

AIM: To analyze the percentages of hepatocytes with increased nuclear DNA content, i.e., tetraploid (4n) and octoploid (8n) nuclei, and then compared mononuclear and binuclear hepatocyte populations.

METHODS: The percentages of mononuclear diploid (2n), 4n, and 8n hepatocytes and those of binuclear 2 × 2n, 2 × 4n, and 2 × 8n hepatocytes were determined with a method that can simultaneously measure hepatocyte nuclear DNA content and binuclearity in 62 patients with chronic hepatitis B or C. The percentage of 4n and 8n hepatocytes in the mononuclear hepatocyte population was compared with the percentage of 2 × 4n and 2 × 8n hepatocytes in the binuclear hepatocyte population.

RESULTS: The percentages of 4n and 8n hepatocytes in mononuclear hepatocytes and 2 × 4n and 2 × 8n hepatocytes in binuclear hepatocytes were similar, regardless of the activity or fibrosis grade of chronic hepatitis and regardless of the infecting virus.

CONCLUSION: The distribution of nuclear DNA content within mononuclear and binuclear hepatocyte populations was conserved during the course of chronic viral hepatitis.

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Key words: Chronic viral hepatitis; Hepatocyte binuclearity; Hepatocyte ploidy; Nuclear DNA content

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INTRODUCTION

Hepatocyte polyploidization and binucleation are two important features of liver growth and physiology. In adult humans, the number of polyploid liver cells is reportedly 20%-45%[1,2]. The importance of controlling hepatocyte polyploidization in both normal and pathological liver conditions has been reported. During postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization[3]. In adults, hepatocyte polyploidization is differentially regulated upon loss of liver mass and liver damage. Partial hepatectomy induces marked cell proliferation followed by an increase in hepatocyte ploidy[4]. In contrast, in different liver pathologies, such as hepatocellular carcinoma, growth shifts to a non-polyploidizing pattern and expansion of the diploid hepatocyte population is observed[5].

We previously studied changes in hepatocyte ploidy and binuclearity profiles in patients with chronic viral hepatitis and found that the percentage of diploid hepatocytes was significantly reduced in patients with high hepatitis activity and marked fibrosis and that the polyploid hepatocyte fraction was increased in these patients[6]. Thus, during chronic hepatitis, the changes in ploidization is similar to that observed after partial hepatectomy in adults. In this previous study, we validated a technique that allows simultaneous measurement of hepatocyte nuclear DNA content and hepatocyte binuclearity in the same liver sections. This is a technique that makes it possible to evaluate the DNA content of a binuclear hepatocyte.

In the present study, we used the same technique to analyze the nuclear DNA contents of mononuclear and binuclear hepatocytes, to compare the changes in the distribution of hepatocytes with different nuclear DNA content between mononuclear and binuclear hepatocyte populations during the course of human chronic hepatitis.

MATERIALS AND METHODS

Subjects

A total of 62 patients with chronic viral hepatitis (44 men

and 18 women, mean age 39.6 ± 5.7 years) were studied. An ultrasonography-guided needle liver biopsy had been performed on all patients between March 1996 and March 2001. Twenty-seven patients were chronically infected with hepatitis B virus (HBV), whereas the other 35 patients were infected with hepatitis C virus (HCV). On the basis of histological findings, hepatitis activity as determined with the METAVIR scoring system[7] was A0 in 15 patients, A1 in 13 patients, A2 in 13 patients, and A3 in 21 patients, and the degree of fibrosis was F0 in 7 patients, F1 in 21 patients, F2 in 11 patients, and F3 in 23 patients.

Methods

Analyses of hepatocyte nuclear DNA content and hepatocyte binuclearity were performed as described previously[6,8,9]. Three-micrometer-thick, paraffin-embedded liver tissue sections were incubated for 1 h with anti-cytokeratin antibody (1:50, KL1, Immunotech, S.A., Marseille, France) as the primary antibody for immunostaining of hepatocyte membrane, followed by a 30-min incubation with biotinylated swine anti-goat, mouse, rabbit immunoglobulin solution (1:200, Dako) and a 30-min incubation with FITC-conjugated streptavidin (1:200, Dako). Sections were then stained for 20 min with Hoechst 33342 (1 mg/L) to stain DNA. Tissue sections were then examined under a Zeiss inverted microscope (Axiovert 35, Carl Zeiss, Gottingen, Germany) equipped for epi-illumination. Images were captured with a cooled, charged coupled device (CCD) camera (KAF 1400-G2, class 2, Photometrics, Tucson, AZ) on 4056 grey levels. Automatic quantitative image analysis was performed in 12 bits with the IPLab Spectrum version 3.1 software. Hepatocytes were classified as mono- or binuclear hepatocytes on the basis of comparisons of fluorescent and membrane labelling images. Other liver cell types, which were defined by morphologic characteristics, were eliminated. Integrated fluorescence intensity was stored in computer files for analysis. A histogram of the fluorescence intensity per section was drawn, and the distribution of each hepatocyte population (2n [diploid] DNA content as the first peak on the histogram, 4n [tetraploid] DNA content as the second peak on the histogram, and 8n [octoploid] DNA content as the third peak on the histogram) was calculated. To develop a histogram of the fluorescence intensity of binuclear hepatocytes, we used the fluorescence intensity of the nucleus with the clearest edge when only one of the two nuclei of a binuclear hepatocyte had a clear edge on the image analyzed. When two nuclei had clear edges, we calculated the average of fluorescence intensity of the two nuclei and used this value as fluorescence intensity of the hepatocyte. A minimum of 300 hepatocytes on eight to 12 separate fields was studied. To ensure that the same cells were not counted twice, slides were read by two observers in a systematic manner of moving from right to left along the slide and then along successive descending lines. All analyses were performed blind to any sample-specific data.

Correlations between values were analyzed by Spearman test, and a $P < 0.05$ was accepted as statistically significant. The study was approved by the Institutional Review Board of Hopital Necker-Enfants Malades (Paris, France) and was carried out in accordance with the Helsinki declaration.

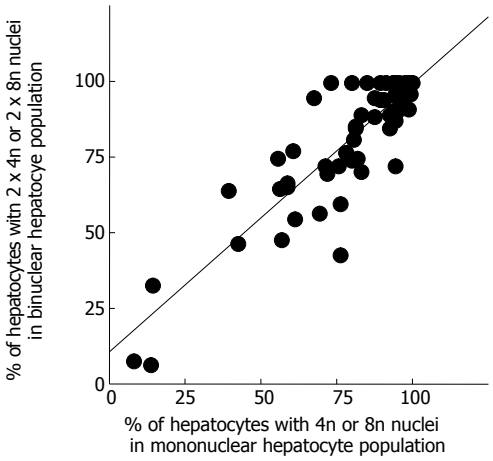

Figure 1 Correlation between percentage of hepatocytes with 4n or 8n nuclei in mononuclear hepatocyte population and percentage of hepatocytes with 2 x 4n or 2 x 8n nuclei in binuclear hepatocyte population. [$P < 0.0001$; correlation coefficient, 0.826 (0.726-0.892)].

RESULTS

We analyzed the percentages of binuclear hepatocyes with diploid (2n) nuclei and those with tetraploid (4n) or octoploid (8n) nuclei (namely binuclear 2 × 2n and 2 × 4n + 2 × 8n) and compared them with the percentages of mononuclear hepatocyte with 2n nucleus and those with 4n or 8n nucleus (namely mononuclear 2n and 4n + 8n) in samples from patients with chronic viral hepatitis. We found a significant correlation between the percentages of mononuclear 4n and 8n hepatocytes and binuclear 2 × 4n and 2 × 8n hepatocytes ($P < 0.0001$; correlation coefficient, 0.826 [0.726-0.892]); the percentages of nuclei of 4n or 8n DNA content was maintained in the mononuclear hepatocyte population and in the binuclear hepatocyte population (Figure 1). This correlation was maintained when we focused specifically on patients with HBV or HCV. The correlation was also maintained regardless of activity of hepatitis, grade of liver fibrosis, age, or sex (data not shown). In contrast, there was no correlation between the percentage of binuclear 2 × 4n and 2 × 8n hepatocyes and the percentage of binuclear hepatocytes in the total hepatocyte population ($P = 0.1276$; correlation coefficient, 0.196 [-0.057-0.425]; Figure 2). The percentage of binuclear hepatocytes with increased nuclear DNA content (2 × 4n and 2 × 8n hepatocytes), therefore, was not associated with the percentage of binuclear hepatocytes in the total hepatocyte population.

DISCUSSION

Flow cytometry can measure nuclear DNA content but cannot distinguish between nuclei of mononuclear hepatocytes and those of binuclear hepatocytes. In addition, morphologic observation of hematoxylin and eosin-stained sections can distinguish mononuclear and binuclear hepatocytes but cannot quantify nuclear DNA content. To our knowledge, the present study is the first to compare the distributions of nuclear DNA content between mononuclear and binuclear hepatocyte populations with a novel method that allowed evaluation of the distribution of nuclear DNA content (2 ×

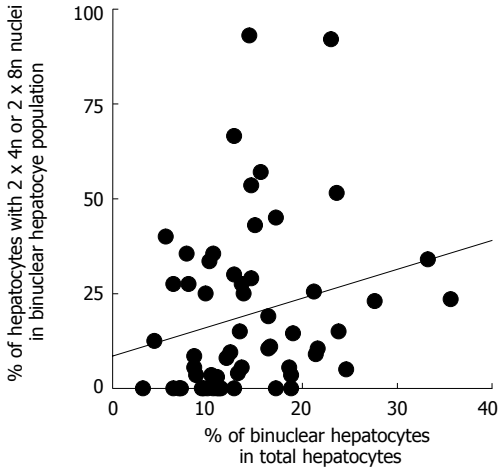

Figure 2 Correlation between percentage of hepatocytes with 2 x 4n or 2 x 8n nuclei in binuclear hepatocyte population and that of binuclear hepatocytes as a portion of total hepatocytes. [P = 0.1276; correlation coefficient, 0.196 (-0.057-0.425)].

2n, 2 × 4n, and 2 × 8n) exclusively in binuclear hepatocytes.

With respect to the hypothesis that mononuclear hepatocytes are stimulated to form binuclear hepatocytes, our findings suggest that both diploid and polyploid mononuclear hepatocytes are similarly stimulated to form binuclear hepatocytes, suggesting the possibility that the formation of binuclear hepatocytes is regulated by a single factor for both diploid and polyploid hepatocytes. Alternatively, it is possible that both diploid and polyploid hepatocytes are sensitive to a single stimulus for binuclear hepatocyte formation. This appears to be independent of viral infection and is maintained throughout the course of chronic hepatitis.

In a previous report[6], we showed that the decrease in the percentage of diploid hepatocytes was correlated with the progression of chronic hepatitis. In addition, we showed there was a higher percentage of binuclear hepatocytes in patients with HBV infection than those with HCV infection. Our present results indicate that the percentage of diploid hepatocytes decreases in both mononuclear hepatocyte and binuclear hepatocyte populations, in a similar way according to the progression of chronic hepatitis. Also, the present data indicate that binuclear hepatocytes increase in patients infected with HBV, conserving the distribution of different nuclear DNA content (2 × 2n, 2 × 4n, and 2 × 8n hepatocytes).

The mechanisms that underlie hepatocyte polyploidization are still largely unknown. The importance of binuclear hepatyocyte formation as a step towards hepatocyte polyploidization has been reported. We previously provided direct evidence for the pivotal role of binuclear hepatocytes in the formation of 4n mononuclear hepatocytes, indicating a close association between binuclear hepatocyte formation and polyploid mononuclear hepatocyte formation *in vitro*[9]. Our present study indicated that formation of polyploid mononuclear hepatocytes is associated with formation of polyploid binuclear hepatocyte in human chronic viral hepatitis. Further studies are needed to clarify the mechanisms that control hepatocyte polyploidization and binucleation during human chronic viral hepatitis. In addition, the influence of steatosis, which is often observed in case of chronic hepatitis C and can reduce replication in hepatocytes, on hepatocyte polyploidization and binucleation should be elucidated in the future.

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RAPID COMMUNICATION

Functional activity of the rectum: A conduit organ or a storage organ or both?

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Abstract

AIM: To investigate whether the degree of rectal distension could define the rectum functions as a conduit or reservoir.

METHODS: Response of the rectal and anal pressure to 2 types of rectal balloon distension, rapid voluminous and slow gradual distention, was recorded in 21 healthy volunteers (12 men, 9 women, age 41.7 ± 10.6 years). The test was repeated with sphincteric squeeze on urgent sensation.

RESULTS: Rapid voluminous rectal distension resulted in a significant rectal pressure increase ($P < 0.001$), an anal pressure decline ($P < 0.05$) and balloon expulsion. The subjects felt urgent sensation but did not feel the 1st rectal sensation. On urgent sensation, anal squeeze caused a significant rectal pressure decrease ($P < 0.001$) and urgency disappearance. Slow incremental rectal filling drew a rectometrogram with a "tone" limb representing a gradual rectal pressure increase during rectal filling, and an "evacuation limb" representing a sharp pressure increase during balloon expulsion. The curve recorded both the 1st rectal sensation and the urgent sensation.

CONCLUSION: The rectum has apparently two functions: transportation (conduit) and storage, both depending on the degree of rectal filling. If the fecal material received by the rectum is small, it is stored in the rectum until a big volume is reached that can affect a degree of rectal distension sufficient to initiate the defecation reflex. Large volume rectal distension evokes directly the rectoanal inhibitory reflex with a resulting defecation.

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Key words: Anal pressure; Rectal pressure; Rectometry; First rectal sensation

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INTRODUCTION

The rectum, a continuation of the colon, acts to propel the stools to the exterior and is thus considered as a conduit[1-3]. However, its function as a conduit for stools is controversial[4-6]. Stools are palpable on digital rectal examination (DRE) in many healthy volunteers who do not feel the desire to defecate[4-6]. Neil and Rampton[5] reported that stools are present in the rectum in 31% of normal subjects. Stools are also palpated by DRE in the lower rectum of healthy subjects and appear in the radiograms. Our studies have shown that the rectum stores the stools if the quantity is small, while functions as a conduit if the stools received from the sigmoid colon are voluminous. A small stool volume reaching the rectum from the sigmoid colon probably does not affect rectal distension or evoke the rectoanal inhibitory reflex (RAIR). In such case the stools accumulate in the rectum until they reach a big distending volume that initiates the RAIR. On the other hand, big distending volumes of the stools propelled from the sigmoid colon appear to be able to initiate the RAIR and expel the stools without storage in the rectum. Accordingly, the rectal function as a storage organ or a conduit appears to depend on the volume of fecal matter passing from the sigmoid colon to the rectum and consequently on the degree of rectal distension the volume creates.

We thus hypothesized that rectal function as a conduit or reservoir would depend on the degree of rectal distension. This hypothesis was investigated in the current study.

MATERIALS AND METHODS

Subjects

Twenty-one healthy volunteers (12 men, 9 women, mean

age 41.7 ± 10.6 years, range 29-56 years) participated in the study. They were fully informed about the nature of the study, the tests to be done, and their role in the study. They had no anorectal complaint either in the past or at the time of enrolment. The mean stool frequency was 8.6 ± 1.2 times per week (range 8-11), being in accord with that of normal volunteers in our laboratory. Physical examination, including neurological assessment, was normal. Laboratory work was unremarkable. Sigmoidoscopy assured that the rectum was normal in all the subjects. The subjects gave an informed consent, and the study was approved by the Review Board and Ethics Committee of the Cairo University Faculty of Medicine.

Methods

The subject was instructed to fast for 12 h before the test, and the bowel was evacuated by saline enema. The rectum was distended by a thin polyethylene infinitely compliant balloon, 3 cm in diameter, which was attached to the end of a 10 F tube (London Rubber Industries Ltd, London, UK). The pressure measured within the balloon was considered to be representative of the rectal pressure. With the subject lying in the left lateral position and under no medication, the collapsed balloon was introduced into the rectum through the anus. The tube was connected to a strain gauge pressure transducer (Statham 23 bb, Oxnard, CA).

The anal and rectal pressures were separately measured with a saline-perfused tube. The 10 F tube with multiple side ports at its distal closed end was introduced through the anus for 2-3 cm to lie in the rectal neck (anal canal). Another tube was introduced for 8-10 cm to lie in the rectum. The tube was connected to a pneumohydraulic capillary infusion system (Arndorfer Medical Specialities, Greendale, WI), supplied with a pump that delivers saline solution continually via the capillary tube at a rate of 0.6 mL/min. The transducer outputs were registered on a rectilinear recorder (RS-3400, Gould Inc, Cleveland, OH). Occlusion of the recording orifice produced a pressure elevation of greater that 250 cm H_2O/s.

Prior to anal and rectal pressure recording, the gut was allowed a 20-min period to adapt to the rectal balloon and the manometric tubes in the anal canal and rectum. We recorded the response of the rectal and anal pressures to 2 types of rectal balloon distension: rapid voluminous distension and slow gradual distension. The rectal balloon was filled with 200 mL of normal saline in 10 s for rapid filling, and with increments of 20 mL in 10 s for slow filling. The test was performed at rest and while the anal sphincters were squeezed. The subjects were asked to report the onset of awareness of "something" in the rectum (the first rectal sensation) and the urgent sensation to defecate.

To ensure reproducibility of the results, the tests were repeated at least twice in the individual subjects and the mean value was calculated. The results were analyzed statistically by the Student's *t* test and the values were given as mean ± SD.

RESULTS

The study was completed in all the subjects with no adverse side effects. The mean resting rectal pressure was 8.7 ± 0.9 cm H_2O (range 8-10) and the mean anal pressure was 73.6 ± 4.6 cm H_2O (range 65-79).

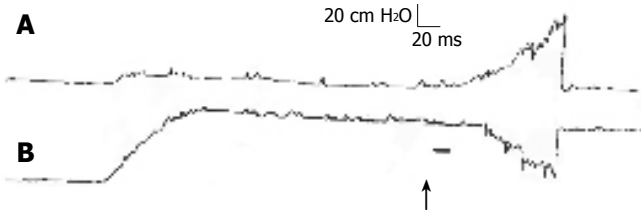

Figure 1 Pressure tracing showing the rectal (**A**) and anal (**B**) pressure response to rapid balloon rectal distension with 200 mL of normal saline in 10 seconds. ↑ = rectal balloon distension.

Rectal and anal pressure response to rapid voluminous rectal distension

Rectal balloon distension with 200 mL of normal saline in 10 s resulted in a significant increase of the rectal pressure and a decrease of the anal pressure (Figure 1).The balloon was expelled to the exterior. The mean rectal pressure was 63.4 ± 8.2 cm H_2O (range 56-82 cm H_2O, $P < 0.001$) and the mean anal pressure was 22.4 ± 2.6 cm H_2O (range 18-26 cm H_2O, $P < 0.01$). The subjects felt the urgent sensation followed by balloon expulsion, but did not feel the first rectal sensation. When the subject upon feeling the urgent sensation, was asked to squeeze the anal sphincters for seconds, the mean anal pressure rose to 146.8 ± 16.2 cm H_2O (range 128-166 cm H_2O), resulting in disappearance of the urgent sensation and a significant decrease in the rectal pressure to 10.8 ± 1.1 cm H_2O (range 8-12 cm H_2O, $P < 0.001$). The balloon was not expelled but stored in the rectum. After a few seconds however, if the patient was asked not to squeeze the sphincters, the urgent sensation recurred and the balloon was expelled.

The test was reproducible with no significant difference ($P < 0.05$) in the recorded pressure values. There was also no significant difference between men and women.

Rectal and anal pressure response to slow incremental rectal filling

During rectal filling with a small volume (20 mL in 10 s), rectometrogram was produced (Figure 2). The curve showed a "tone limb" representing the rectal pressure as it increased during rectal filling, and an "evacuation limb" representing the rectal pressure during the process of balloon expulsion. The tone limb carried the points of incidence of the first rectal and urgent sensations while displaying a gradual incline until expulsion started.

The evacuation limb described a "curve" which was continuous with the tone limb. It was manifested at a variable distance after the urgent sensation and depended on the subject's desire to evacuate. The evacuation curve had an ascending limb rising slowly as a continuation of the tone limb, and a vertical descending limb (Figure 2).

The mean rectal balloon distending volume was 78.3 ± 16.6 mL (range 53-108 mL) at the first sensation and 152.6 ± 18.9 mL (range 106-182 mL) at the urgent sensation. The mean rectal pressure was 51.7 ± 9.8 cm H_2O at the 1st rectal sensation and 62.9 ± 14.2 cm H_2O at the urgent

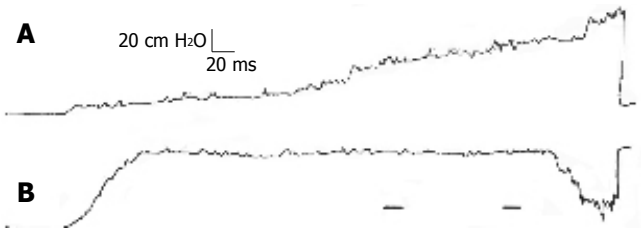

Figure 2 Pressure tracing (rectometrogram) showing the rectal (**A**) and anal (**B**) pressure response to balloon rectal distension in increments of 20 mL in 10 seconds.

Table 1 Anal and rectal pressures at the 1st rectal sensation and urgent sensation on slow gradual rectal filling (mean ± SD)

Pressure (cm H_2O)				
	Rectal		Anal	
	Mean	Range	Mean	Range
Basal	8.7 ± 0.9	8-10	73.6 ± 4.6	65-79
1st rectal sensation	51.4 ± 9.8[d]	32-68	70.6 ± 5.2	61-78
Urge	62.9 ± 14.2[d]	45-86	18.7 ± 2.3[b]	16-24

[b]$P < 0.01$ *vs* the basal values; [d]$P < 0.001$ *vs* the basal values.

sensation, while the mean anal pressure was 70.6 ± 5.2 cm H_2O and 18.7 ± 2.3 cm H_2O, respectively (Table 1).

The aforementioned results were reproducible with no significant difference when the test was repeated in the individual subjects.

DISCUSSION

The rectum presumably receives stools from the sigmoid colon, either in a big volume affecting rapid voluminous distension of the rectum, or in small masses affecting gradual rectal distension. The current study could shed some light on the rectal function during the rapid voluminous distension and the slow gradual rectal distension.

Rectum as a conduit

Rapid voluminous rectal distension seems to evoke the recto-anal inhibitory reflex as is evident from the increased rectal and decreased anal pressures as well as from balloon expulsion. The subjects feel the urgent sensation but not the first rectal sensation which is normally felt in the gradual rectal distension. In such conditions, the rectum is considered as a conduit, conducting the stools directly from the colon to the exterior. It is likely that the big voluminous rectal distension induces stimulation of the rectal mechanoreceptors with a resulting initiation of the defecation reflex. In this case the rectum evacuates the received stools and remains empty, provided the circumstances are opportune for defecation.

Squeeze of the anal sphincters could abort the urgent sensation and affect waning of the desire to defecate. The mechanism needs to be further discussed. If circumstances are inopportune for defecation, sphincteric squeeze causes rectal relaxation mediated by the voluntary anorectal inhibition reflex[7]. However, after some time, the loaded rectum re-contracts probably due to re-stimulation of the rectal mechanoreceptors. If the desire to defecate is still opposed, the external sphincter contracts again. This process is repeated till either defecation is acceded to, or a prolonged contraction of the un-striped rectal detrusor tires out the striped short-contracting external anal sphincter which involuntarily relaxes leading to internal anal sphincter relaxation and opening of the rectal neck.

Rectum as a reservoir

Slow incremental rectal filling does not affect rectal contraction until rectal filling reaches a large enough volume to stimulate the rectal mechanoreceptors and evoke the defecation reflex. During the period of slow gradual filling, the rectum presumably contains stools which are commonly palpated in some subjects during DRE. We postulate that in such cases the amount of stools received from the sigmoid colon is too small to stimulate the rectal mechanoreceptors or evoke the defecation reflex. The stools most probably continue to pile up in the rectum until an adequate volume is reached that stimulates the rectal mechanoreceptors and evokes the recto-anal inhibitory reflex. In such conditions, the stools may remain accumulated in the rectum for an extended period during which the rectum acts as a "storage" organ. We speculate that stool storage in the rectum may affect its consistency and act as a contributing factor in the genesis of constipation. The storage function of the rectum is apparently due to the adaptability of its smooth muscle to small volume rectal distension. This mechanism of "receptive relaxation" is similar to that occurring in the stomach.

In conclusion, the rectum has apparently 2 functions: transportation (conduit) and storage, both depending on the degree of rectal distension. If the fecal material received by the rectum is small, it is stored in the rectum until a big volume accumulated affects rectal distension sufficient to initiate the defecation reflex. However, stool storage in the rectum may be a contributing factor in the genesis of constipation. Large volume rectal distension evokes directly the recto-anal inhibitory reflex with a resulting defecation.

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RAPID COMMUNICATION

Azithromycin in one week quadruple therapy for *H pylori* eradication in Iran

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Abstract

AIM: To investigate eradication rates, patient compliance and tolerability of a 1-wk Azithromycin-based quadruple therapy versus the 2-wk conventional therapy.

METHODS: A total of 129 *H pylori*-positive patients were randomized to either omeprazole 20 mg, bismuth subcitrate 240 mg, azithromycin 250 mg, and metronidazole 500 mg, all twice daily for 1-wk (B-OAzM) or omeprazole 20 mg, bismuth subcitrate 240 mg, amoxicillin 1g, and metronidazole 500 mg all twice daily for 2-wk (B-OAM). *H pylori* infection was defined at entry by histology and rapid urease test and cure of infection was determined by negative urea breath test.

RESULTS: *H pylori* eradication rates produced by B-OAzM and B-OAM were 74.1% and 70.4% respectively based on an intention to treat analysis, and 78.1% versus 75.7% respectively based on a per-protocol analysis. The incidence of poor compliance was lower, although not significantly so, in patients randomized to B-OAzM than for B-OAM (3.5% versus 4.3%) but intolerability was similar in the two groups (35% versus 33.3%).

CONCLUSION: 1-wk azithromycin based quadruple regimen achieves an *H pylori* eradication rate comparable to that of standard 2-wk quadruple therapy, and is associated with comparable patient compliance and complications.

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Key words: Peptic ulcer; Treatment; Azithromycin; *H pylori*; Non-ulcer dyspepsia

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INTRODUCTION

H pylori is a common human pathogen that has been shown to be a major factor in peptic ulcer disease. It has also been linked to gastric adenocarcinoma and gastric lymphoma[1].

A number of antimicrobial agents have been used in various regimens to eradicate *H pylori*. Clinical trails are regularly undertaken to search for simpler but equally effective regimens[2-4]. Azithromycin, a new generation macrolide, has some special attributes that suggest it would be a promising compound to be used in regimens for *H pylori* eradication. It is acid-stable, has a long half-life and achieves remarkably high concentration in the gastric tissue[3,5]. There have been several clinical trials of azithromycin for the therapy of *H pylori* infection[3,4,6,7]. As the pharmacological properties of azithromycin make it possible to use shorter courses, the problem has been to define an optimal dose and duration of azithromycin treatment in the course of therapy[3,4]. On the other hand, in developed countries, regimens of one week's duration are recommended for *H pylori* eradication[1,2] but in developing countries 1-wk regimens failed to eradicate the microbe. A minimum of 10 to 14 d of treatment were needed for eradication of the microbe, even in the presence of a favorable sensitivity profile[8-10]. The aim of this study was to assess the efficacy of azithromycin in a 1-wk regimen compared with a conventional 2-wk regimen in Iran, so we compared two quadruple regimens; bismuth subcitrate, omeprazole, azithromycin, and metronidazole, for 1-wk (B-OAzM) and bismuth subcitrate, omeprazole, amoxicillin and metronidazole, for 2-wk (B-OAM) in *H pylori* eradication. The safety and tolerability of the two drugs combinations were also evaluated and compared.

MATERIALS AND METHODS

Patients considered for the study included individuals 18-80 years old with upper GI symptoms that were referred to our gastroenterology clinic for upper endoscopy. Patients with *H pylori* infection were included in the study. Other inclusion criteria included indication for treatment as per the National Institutes of Health (NIH)

Table 1 Clinical and demographic data of patients in the treatment groups

Data	B-OAM-2 wk regimen *n* (%)	B-OAzM-1 wk regimen *n* (%)	*P* value[1]
ITT analysis	71	58	
Age: yr, Mean (SD)	48.3 (7.4)	46.7 (5.4)	0.17
Male	45 (63.3)	33 (56.8)	0.45
NSAID users	21 (29.5)	15 (25.8)	0.63
Cigarette smokers	11 (15.4)	8 (13.7)	0.78
Abdominal pain	47 (66.1)	31 (53.4)	0.14
Heartburn	14 (19.7)	18 (31)	0.13
Dyspepsia	57 (80.2)	40 (68.9)	0.13
PU	37 (52.1)	25 (43.1)	0.3
Non-ulcer dyspepsia	34 (47.8)	33 (56.8)	0.3
Loss to follow up	2	1	
Poor compliance	3	2	
Drop-outs	5	3	
PP analysis	66	55	

[1]*t*; for means and χ^2 for proportions, ITT; intention to treat, PP; per protocol.

Consensus Conference including: peptic ulcer disease, history of peptic ulcer, chronic gastritis, gastric mocusa associated lymphoid tissue, or intestinal metaplasia[1,7]. Between October 2003 and October 2004, a total of 129 patients were enrolled in the study. All patients gave written informed consent before entering the study and the protocol was reviewed and approved by the Semnan Gastrointestinal and Liver diseases Research Center. The criteria for exclusion were: intake of proton pump inhibitors, antibiotic or bismuth salts within 4 wk prior to the study, stomach surgery, known hypersensitivity to one of the study medications, patients with liver cirrhosis, renal failure or other serious severe concomitant illness, pregnant women, and patients who had previously undergone eradication therapy. These criteria were ascertained by taking a complete history, physical examination, and appropriate hematological and biochemical tests. The demographic and endoscopic data of these patients are reported in Table 1.

On initial endoscopy, *H pylori* infection was deter-mined by rapid urease test and histological assessment. Rapid urease test was performed using two biopsy specimens; one from the antrum and the other from the corpus[11]. Histological assessment of *H pylori* status was performed using one further biopsy specimen from the antrum; within 3 cm of the pylorus (hematoxylin-eosin and Giemsa stains)[12]. The patients were considered to be infected if both the urease test and histology were positive on initial testing. The patients that satisfied the inclusion criteria were randomly assigned to one of the following regimes; one group received a regimen of bismuth subcitrate 240 mg, omeprazole 20 mg, azithromycin (Azithromycin TC ®, Tehran shimi, Iran) 250 mg and metronidazole 500 mg, all bid, for 1-wk (B-OAzM) and the second group received a regimen of bismuth subcitrate 240 mg, omeprazole 20 mg, amoxicillin 1 g and metronidazole 500 mg, all bid, for 2-wk (B-OAM). Patients with duodenal or gastric ulcers continued on omeprazole (20 mg/d) for a total of 1 mo. Repeat examination was performed 1 wk and 4-8 wk after the cessation of therapy. Subjects recorded any side effects and change in symptoms, and performed an exit interview and pill count to evaluate compliance and side effects. Hematological and biochemical analyses were performed at the last visit. *H pylori* infection was determined by urea breath test (UBT) at the second examination. All patients were instructed to discontinue all proton pump inhibitors, H2 blockers, and bismuth for at least 4 wk before UBT. Eradication was defined on the basis of a negative UBT (carbon 13-Isomax 2000 TM device); results under the 5 cut off were considered negative[13].

Analysis of the two groups was conducted in the form of both per protocol (PP) and intention to treat (ITT). The ITT analysis for eradication was defined before the study to include all subjects who were randomized and received at least one dose of medication. The PP analysis for eradication included all subjects who took at least 80% of each study medication as prescribed.

The comparison of efficacy was evaluated using the χ^2 test. The analysis was performed using the SPSS 11.5 statistical package (SPSS, Chicago, IL).

RESULTS

The two groups were comparable in terms of common clinical variables that are summarized in Table 1. Three patients were lost to follow up (two from the B-OAM regimen and one subject from the B-OAzM regimen); also five patients discontinued the drugs because of severe side effects (three from the B-OAM regimen and two subject from the B-OAzM regimen). The remaining 121 patients completed the study as planned.

PP analysis: *H pylori* infection was eradicated in 43 of 55 patients in the B-OAzM group (78.18%, CI0.95: 64.98%-88.18%) and in 50 of 66 patients in the B-OAM group (75.75%, CI0.95: 63.63%-85.46%); the difference was not statistically significant ($\chi^2 = 0.1$, $P = 0.75$).

In patients with peptic ulcer, *H pylori* infection was eradicated in 19 of 25 patients in the B-OAzM group (76%, CI0.95: 54.87%-90.64%) and in 28 of 37 in the B-OAM group (75.67%, CI0.95: 58.8%-88.22%); the difference was not statistically significant ($\chi^2 < 0.001$, $P = 0.97$).

ITT analysis: *H pylori* infection was eradicated in 74.13% (CI0.95: 60.95%-84.74%) in the B-OAzM group and 70.42% (CI0.95: 58.4%-80.67%) in the B-OAM group; the difference was not statistically significant ($\chi^2 = 0.3$, $P = 0.58$).

Complications: the overall results for side effects are summarized in Table 2. Complications were noted in 14 patients in the B-OAzM group (25.45%) and 17 patients in the B-OAM group (25.75%) with no statistically significant differences between the groups ($\chi^2 < 0.001$, $P = 0.96$). The symptoms were mild and did not necessitate any additional treatment except in the five patients that discontinued the drugs for severe complications.

DISCUSSION

Although various regimens have been recommended for *H pylori* eradication, all of them include at least 2 antibiotics and one acid inhibitory drug[1]. In western countries,

Table 2 Complications of drugs in *H pylori* treatment

Complication	B-OAM-2 wk regimen	B-OAzM-1 wk regimen	*P* value[2]
n	69[3]	57[3]	
Diarrhea (%)	4 (5.7)	4 (7)	
Vomiting (%)	5 (7.2)[1]	4 (7)[1]	
Abdominal pain (%)	7 (10.1)	5 (8.7)	
Bad taste (%)	7 (10.1)	5 (8.7)	
Anal pain (%)	0	2 (3.5)	
Total (%)	23 (33.3%)	20 (35%)	0.83

[1]Three patients of group "A" and two patients of group "B" excluded from the study for severe vomiting.; [2] χ^2; [3] All patients except lost to follow up cases.

the most effective and usual regimens for preliminary treatment include; triple regimens for at least 1 wk, and use of clarithromycin as the antibiotic of choice against *H pylori*. Metronidazole, on the other hand, has largely been eliminated from first-line *H pylori* therapy because of its intolerability and high drug resistance[14]. However, in developing countries, effective treatments for *H pylori* vary from those used in developed countries. For example in middle east countries it has been shown that one week regimens fail to eradicate the microbe and the course of treatment should be continued for at least 10-14 d to provide for eradication[8-10]. Further, clarithromycin is not appropriate use because of its high price, drug resistance and unavailability[15,16], and so metronidazole is a common and effective drug in *H pylori* treatment in this setting.

Although the prevalence of *H pylori* strains that are resistant to metronidazole varies from 46%-51% in Iran[15-17], it has been shown that this drug in high doses (> 1 g) and in combination with other drugs remains effective against *H pylori*[18-21]. Therefore, in Iran as also done in some Asian countries[14,18] metronidazole is a very common drug used in *H pylori* eradication regimens[22]. In Iran, the most common regimens for first-line treatment are 2-wk quadruple regimens that include; metronidazole, omeprazole, bismuth and tetracycline or amoxicillin[22]. This conventional regimen introduced initially by Hosking, Deboer, Borody, and Laine as an effective regimen in *H pylori* treatment has confirmed efficiency of a 63%-93% eradication rate[7,23-25]. On other hand, due to high rates of resistance to metronidazole, furazolidine was used instead of it, as first or particularly second line treatment[26].

Although 2 wk regimens have been effective in *H pylori* eradication, some patients withdraw from their treatment because of the long duration and large number of tablets. Thus, in this study we showed that a quadruple regimen, where azithromycin replaced amoxicillin, the duration of the treatment can be decreased without any change in its effectiveness.

Azithromycin is a new macrolide related to clarithromycin with an effective role in *H pylori* eradication and it was reported that azithromycin has a synergic effect with esomeprazole, even in presence of drug resistance[27]. In various studies the effectiveness of azithromycin has been evaluated in different doses from 500 mg to 3 g[2,27-29], although in most studies this drug was used for 3 d (1.5 g) as in respiratory tract infection but at this dose it has less effect than clarithromycin[3,5]. However, by increasing the total dose to 3 g, it has been shown that the effectiveness of azithromycin can be restored[3,4,6,7].

This is the first study in which azithromycin was used at a prescribed amount of 3.5 g (250 mg bid for 7d) in the treatment of *H pylori* infection. Fortunately; the patients' tolerance was excellent. There were a few side effects based on biochemical tests, but most side effects were mild and disappeared with conservative therapy without the need to terminate the treatment.

Finally, in areas where clarithromycin cannot be used because of drug resistance or unavailability, azithromycin with a total dose of 3.5 g is an appropriate and safe drug for *H pylori* eradication regimen.

The effectiveness of furazolidone based *triple or quadruple regimens* has been evaluated in Iran[15,22,25,26], but because of resistance to metronidazole, it seems possible that a combination of azithromycin and furazolidone instead of azithromycin and metronidazole will achieve more favorable eradication rates, although further evaluation is needed.

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RAPID COMMUNICATION

Role of a probiotic (*Saccharomyces boulardii*) in management and prevention of diarrhoea

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Abstract

AIM: To assess the efficacy and safety of Saccharomyces boulardii (*S. boulardii*) in acute watery diarrhoea and its role in reducing the frequency of episodes of diarrhoea in subsequent two months.

METHODS: Children from 2 mo to 12 years of age, with acute diarrhoea were selected according to inclusion criteria and randomised in *S. boulardii* group (treated with ORS, nutritional support and *S. boulardii*, 250 mg bid) and in control group (treated with ORS and nutritional support only). Active treatment phase was 5 d and each child was followed for two months afterwards. Frequency and consistency of stools as well as safety of drug was assessed on every visit. A comparison of two groups was done in terms of number of diarrhoeal episode in subsequent two months.

RESULTS: There were fifty patients in each group. Baseline characteristics such as mean age and the average frequency of stools were comparable in *S. boulardii* and control group at the time of inclusion in the trial. By d 3 it reduced to 2.7 and 4.2 stools per d respectively and by d 6 it reduced to 1.6 (*S. boulardii* Group) and 3.3 (control group). The duration of diarrhoea was 3.6 d in *S. boulardii* group whereas it was 4.8 d in control group (P = 0.001). In the following two months, *S. boulardii* group had a significantly lower frequency of 0.54 episodes as compared to 1.08 episodes in control group. The drug was well accepted and tolerated. There were no reports of the side effects during treatment period.

CONCLUSION: *S. boulardii* significantly reduces the frequency and duration of acute diarrhoea. The consistency of stool also improves. The drug is well-tolerated.

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Key words: Acute watery diarrhoea; Probiotic; *Saccharomyces boulardii*; Frequency of episodes of diarrhoea; Weight gain

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INTRODUCTION

Acute infectious diarrhoeal disease is a worldwide problem with over two million deaths each year. Diarrhoeal diseases are a leading cause of childhood morbidity and mortality and over 200 000 children die every year (600 deaths per day) in Pakistan. Repeated episodes of diarrhoea lead to under-nutrition. In Pakistan, every child gets, on average, 5-6 episodes of diarrhoea per year.

Last couple of decades has seen greater understanding of pathogenesis and simple methods of management of diarrhoea. Various modalities of interventions have been used in different parts of the world to improve the diarrhoeal mortality and morbidity which include oral rehydration salt (ORS), cereal based ORS, antibiotics, anti-diarrhoeals, antispasmodics and anti-emetics. Some of these modalities later proved to have variable harmful effects. These harmful effects include worsening of diarrhoea, increased duration of diarrhoea, adverse effects on gut motility leading to paralytic ileus. In addition, there are other systemic untoward effects[1,2].

Gastrointestinal disease is often a consequence of a myriad of factors, which disturb the bowel's complex ecosystem. The concept of modulating bacterial activities, directed towards improving gut microbial function, has a long history. The use of yoghurt (as probiotic) in the treatment of diarrhoea has been known for a long time. It is now recognised that the most frequently used method of influencing the gut flora composition is that of probiot-

ics. A probiotic is a living micro organism administered to promote the health of the host by treating or preventing infections owing to strains of pathogens[3]. Bio-therapeutic agents are defined as probiotics registered as drugs.

The modern view of probiotic therapy derives from the concept of a well functioning gut barrier and a normal balanced microbiota.

Numerous probiotic agents have been studied for the management of diarrhoeal disease. In particular, the prevention and management of acute viral diarrhoea, the treatment of recurrent *Clostridium difficile* diarrhoea, as well as the control of antibiotic-associated diarrhoea seem to be areas of significant potential benefit. A few agents, including *Lactobacillus GG*, *Lactobacillus reuteri*, and *Saccharomyces boulardii (S. boulardii)*, seem to be promising agents for the amelioration of the course of acute diarrhoea in children when used therapeutically[4]. Amongst these, all are bacteria except *S. boulardii*, which is yeast.

S. boulardii is a non-pathogenic yeast first isolated from lychee fruits in Indonesia and used first in France to treat diarrhoea, in the beginning of the 1950s. A lyophilised form is in clinical use in Europe, Asia, Africa, and Central and South America.

Preclinical and experimental studies of *S. boulardii* have demonstrated an anti-inflammatory, antimicrobial, enzymatic, metabolic and antitoxinic activity. *S. boulardii* secretes a 54-KDa protease which has been shown to neutralize certain bacterial toxins; *S. boulardii* is also able to stimulate an immune response in the intestinal mucosa. It has a trophic effect by enhancing the metabolic function of the mucosa. *S. boulardii* releases polyamines, which are implicated in stimulating the enzymatic activity of the colonic mucosa[3,5].

Based on our previous experience of use of *S. boulardii* in the treatment of diarrhoea, this study was undertaken to assess the efficacy of *S. boulardii* in the treatment of diarrhoea and reoccurrence of diarrhoea in subsequent two months.

MATERIALS AND METHODS

Patients

This randomised controlled clinical trial was carried out at Kharadar General Hospital, Karachi, catering to the needs of approximately one million population and situated in the middle and low-income community. An informed consent was obtained from parent/guardian of every child included in the trial. The children from 2 mo to 12 years of age presenting with acute watery diarrhoea of mild to moderate severity, fulfilling the inclusion criteria were included in this trial.

Children with severe inter-current illnesses, severe diarrhoea and dehydration requiring hospitalisation and intravenous therapy, presenting with temperature above 38.5℃, who were treated by any other anti-diarrhoeal/antibiotics in last 24 h as well as severely malnourished children were excluded from the trial. At inclusion, stool specimen was sent for bacterial culture and sensitivity as well as for Rota virus detection.

The study population of 100 children was randomised into two groups. In *S. boulardii* group, patients were managed by WHO-CDD protocol[6] plus *S. boulardii* (250 mg bid) administered orally diluted in water or other semi-solid food. The product was manufactured by Hilton Pharma (Pvt) Ltd. under the license of Biocodex, France. In the control group patients were managed by WHO-CDD protocol only. The active treatment period was 5 d. Treatment of the subsequent episodes of diarrhoea was at the discretion of the treating physician.

Table 1 Baseline characteristics

Patient characteristics	*S. Boulardii* (n = 50)	Control (n = 50)	P value
Age (mo)			
Mean	18.3	26.01	0.08
SD	20.33	23.37	
Weight (kg)			
Mean	8.2	9.15	0.224
SD	3.58	4.13	
Sex			
Female	50%	50%	1
Male	50%	50%	
Stool culture/Sensitivity	26%		
Bacteria isolated	74%	12%	0.125
Not isolated		88%	
Rota virus			
Positive	16%	20%	0.795
Negative	84%	80%	

All study participants were examined on d 0 (inclusion day), and followed up on d 3 and d 6 during active treatment phase and every month for two months thereafter for observation.

The first visit data collection included date of onset of diarrhoea, previous treatment (where applicable), weight of child, number and consistency of stools, vomiting, body temperature, sign of dehydration and any other data by clinical examination. The second visit information variables included date of stoppage of diarrhoea in case of inter-current recovery, weight of child, daily record of frequency and consistency of stools, tolerance and acceptability of treatment. Similarly on third visit, date of stoppage of diarrhoea in case of inter-current recovery, weight of child, daily record of frequency and consistency of stools, tolerance and acceptability of treatment were recorded in the study record forms.

A monthly observational follow-up data for two months included weight of child at monthly interval and any new episodes of diarrhoea in both the groups.

Statistical analysis

For statistical analysis, t-test was applied to measure the variation in means. $P < 0.05$ was taken as significant.

RESULTS

One hundred patients were analysed in the study, fifty patients in each group. Patient baseline characteristics in control and study groups were comparable (Table 1). Bacteria were isolated in 26% and 12% of the *S. boulardii* and control groups respectively while Rota virus detection

Table 2 Mean numbers of stools and duration of diarrhoea

	S. boulardii	Control	*P* value
Mean number of stools reported on d 0	9.5	8.8	0.37
Mean number of stools reported on d 3	2.8	4.4	0.01
Mean number of stools reported on d 6	1.6	3.3	0.001
Duration of diarrhoea (d)	3.6	4.8	0.001

Table 3 Number of episodes of diarrhoea

	S. boulardii	Control	*P* value
No. of episodes at 1 mo	0.2	0.64	0.001
No. of episodes at 2 mo	0.32	0.56	0.04

Table 4 Weight gain

	Group	% of mean weight gain	*P* value
% increase in wt. at 1 mo	*S. boulardii*	4.4	
	Control	4.2	0.902
% increase in wt. at 2 mo	*S. boulardii*	9.9	
	Control	6.2	0.067

revealed 16% and 20% positive for test and control group respectively.

On d 3 and d 6, there was a significant reduction in reported number of stools in *S. boulardii* group as compared to the control group. The mean duration of diarrhoea was 3.5 d in *S. boulardii* group and 4.8 d in the control group ($P = 0.001$) (Table 2).

Follow-up for the next two months also revealed interesting results. Mean numbers of episodes of diarrhoea by the end of two months, were 0.56 in control group as compared to 0.32 in *S. boulardii* group, which is almost half of that of control group (Table 3).

S. boulardii was well accepted and tolerated by the children and there were no reports of any side effects during the study period.

Table 4 also shows mean weight gain in both groups at mo 1 and 2. Although the difference of gain between the two groups has not reached statistical significance, the percentage of average increase in the experimental group was 9.9% as compared to 6.2% in the control group.

DISCUSSION

There are over $10^{9\text{-}12}$ bacteria per gram of faeces and about 400 different species, more than 10 times the human cells[7]. The intestinal flora is intimately associated with the organ which contains it and with which it forms an ecosystem. Equilibrium within this ecosystem is essential to good health of the individual. It influences the structure, motility, physical and chemical conditions of intestinal tract, metabolic and enzyme activity of mucosa and establishment and maturation of immune system. Finally and above all, the intestinal microbial flora forms a true resistance to colonisation of digestive tract by pathogenic microorganisms. Disruption of intestinal ecosystem occurs in many pathological situations such as infectious diarrhoea or diarrhoea and colitis linked to antibiotic treatment.

Despite awareness about preventive aspects of diarrhoea it remains one of the leading causes of morbidity and mortality in children, because of lack of clean water supply and sanitation.

Search for newer, less harmful agent is continued. Biological agents ("biotherapeutic agents" or "probiotics") have been used to treat a variety of infections, most notably infections of mucosal surfaces such as the gut and vagina. These biotherapeutic agents include certain bacteria and the yeast *S. boulardii*. Given orally, *S. boulardii* seem to be promising agents for the amelioration of the course of acute diarrhoea in children[8].

The current study was based on our previous clinical observation, which revealed that children treated with *S. boulardii* had a decreased number of episodes of diarrhoea in following months.

This study verified our previous observation, as there was a 50% reduction in the number of episodes of diarrhoea in the treatment group as compared to control group (Table 2).

This study also showed a significant improvement in frequency and consistency of stool and reduction in duration of illness in patients who were given *S. boulardii* along with WHO-CDD protocol.

Several studies of *S. boulardii* have been done in children and adults in the treatment of acute diarrhoea[9]. The results of our studies are consistent with some of these studies. However, the current study is the first one to observe the reduction in number of episodes of diarrhoea in the post-treatment follow-up period of two months. Stimulation of local immunity, as demonstrated by the increase of IgA, together with the enhancement of the trophic activity of the mucosa (through the release of polyamines) by *S. boulardii* may, at least in part, explain the long term effect of the yeast.

A meticulous follow-up of the patient resulted in very good compliance. No side effects were observed during the active treatment period with the use of *S. boulardii*. McFarland *et al* also highlight the safety profile in their review on *S. boulardii*[10].

CONCLUSION

Based on our experience of this trial we conclude that *S. boulardii* is a useful and welcome addition to the treatment of acute diarrhoea in children. *S. boulardii* reduces the frequency of stool, and duration of illness. It also reduces the number of episodes of diarrhoea by 50% in the subsequent period of two months.

Though this is the first study of its kind in Pakistan, we are of the opinion that multicenter double blind placebo controlled trials need to be conducted to confirm our observations. Investigators have been using probiotics as

prophylaxis in childhood infection whereby it has been shown to reduce the rate of infection[7,11].

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RAPID COMMUNICATION

Intraoperative endoscopy in obstructive hypopharyngeal carcinoma

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Abstract

AIM: To demonstrate the necessity of intraoperative endoscopy in the diagnosis of secondary primary tumors of the upper digestive tract in patients with obstructive hypopharyngeal carcinoma.

METHODS: Thirty-one patients with hypopharyngeal squamous cell carcinoma had been operated, with radical intent, at our Institution in the period between 1978 and 2004. Due to obstructive tumor mass, in 7 (22.6%) patients, preoperative endoscopic evaluation of the esophagus and stomach could not be performed. In those patients, intraoperative endoscopy, made through an incision in the cervical esophagus, was standard diagnostic method for examination of the esophagus and stomach.

RESULTS: We found synchronous foregut carcinomas in 3 patients (9.7%). In two patients, synchronous carcinomas had been detected during preoperative endoscopic evaluation, and in one (with obstructive carcinoma) using intraoperative endoscopy. In this case, preoperative barium swallow and CT scan did not reveal the existence of second primary tumor within esophagus, despite the fact that small, but T2 carcinoma, was present.

CONCLUSION: It is reasonable to use intraoperative endoscopy as a selective screening test in patients with obstructive hypopharyngeal carcinoma.

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Key words: Hypopharynx; Neoplasms; Squamous cell; Endoscopy; Multiple primary; Synchronous tumors

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INTRODUCTION

The rate of synchronous primary cancers in the upper aerodigestive tract in the reported literature varies widely depending on the population surveyed and the thoroughness of the methods used to evaluate these patients[1]. In 25 studies published over 25 years, the average rate of synchronous primary upper aerodigestive cancers was 4%, ranging from 1.5% to 18%[2]. The relative risk of developing esophageal cancer in patients with head and neck cancer has been reported as being 3-20 times greater than that of control subjects from the general population[3-5]. The phenomenon of neoplastic multicentricity could affect the therapeutic approach, and cause local treatment failure.

The aim of this study was to demonstrate the necessity of intraoperative endoscopy in the diagnosis of secondary primary tumors in upper digestive tract in the patients with obstructive carcinoma of the hypopharynx.

MATERIALS AND METHODS

In the period between January 1st, 1978 and January 1st, 2004, 31 patients with hypopharyngeal squamocellular carcinoma had been operated at the Department of Esophagogastric Surgery, First University Surgical Hospital, Clinical Center of Serbia. In most patients a complete preoperative work-up was performed. Tumor resectability was assessed by means of chest X-ray, barium swallow, esophagoscopy (flexible and rigid), tracheobronchoscopy, ENT evaluation, thoracic and neck CT scan and ultrasonography. In patients with obstructive

lesions, where preoperative esophagogastroscopic evaluation was not feasible, intraoperative endoscopy (with lugol staining method) through cervical esophagotomy was performed (Figure 1). Intraoperative endoscopy was performed in standard manner with diagnostic fiberoptic endoscope. Instrument was introduced in the esophagus through incision in the esophageal wall, made below lower border of the tumor. When tumor involved cervical esophagus and extended to the thoracic inlet, procedure was not feasible.

For the diagnosis of multiple separate (synchronous) primary carcinomas we followed standard criteria[6]: (1) neoplasms must be clearly malignant as determined by histological evaluation; (2) each neoplasm must be geographically separate and distinct. The lesions should be separated by normal-appearing mucosa. If a second neoplasm is continuous to the initial primary tumor, or is separated by mucosa with intraepithelial neoplastic change, two lesions should be considered as confluent growth rather than multicentric carcinomas; (3) the possibility that the secondary neoplasm represents a metastasis should be excluded. The observation that the invasive carcinoma arises from an overlying epithelium, which demonstrates a transition from carcinoma *in situ* to invasive carcinoma is helpful, and when the separate foci have significant difference in histology, the diagnosis of separate primary cancer is appropriate.

RESULTS

Mean age of the patients in our study was 53.5 years (range 36-72 years). The male to female ratio was 1:2.1. The most common complaint was dysphagia (83.9%), indicating an advanced disease. We had no experience with stage Ⅰ and Ⅱ carcinoma. Stage Ⅲ and Ⅳ accounted for 23 (74.2 %) and 8 (25.8%) respectively. Due to obstructive tumor mass, in 7 (22.6%) patients, preoperative contrast radiography was not conclusive, and preoperative endoscopic evaluation could not be performed. Obstructive tumors were predominantly localized in postcricoid region, and mostly infiltrating esophageal ostium. In those patients intraoperative endoscopy, made through incision on the cervical esophagus (below hypopharyngeal tumor), was standard diagnostic method for examination of the esophagus and stomach.

In our series, we found synchronous foregut carcinomas in 3 patients (9.7%), two of them with synchronous carcinoma of the thoracic esophagus, and one with stomach carcinoma. In two patients, synchronous carcinomas had been detected during preoperative endoscopic evaluation, and in one patient (with obstructive carcinoma) using intraoperative endoscopy. In this patient, preoperative barium swallow and CT scan did not detect existence of second primary tumor within esophagus despite the fact that small, but T2 carcinoma, was present.

DISCUSSION

With regards to risk factors for head and neck, or esophageal carcinomas, genetic factors and enviromental factors, such as smoking and alcohol, have been reported to be important[7,8]. These data suggest the concept of "field carcinogenesis"[6]. According to Martins[9] and Kumagai[10] more than 70% of patients with synchronous hypopharyngeal carcinoma have second malignancy in the esophagus, but second gastric malignancy could not be detected. Kodama *et al*[11] also reported high prevalence of synchronous carcinomas of the pharynx and esophagus. Gluckman *et al*[12] recommended the use of panendoscopy in the evaluation of all head and neck cancers. Likewise, McGuirt[13], Leipzig[14], and Shapshay[15] also recommended panendoscopy due to frequent association of head and neck, esophageal and lung carcinomas.

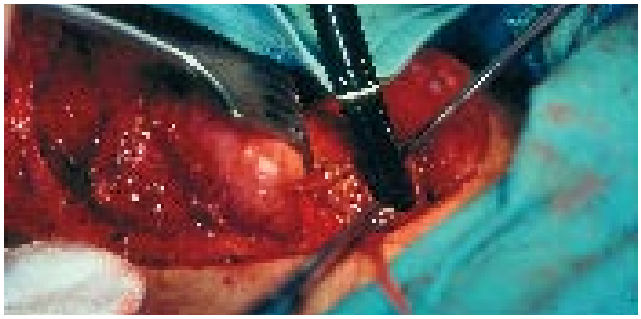

Figure 1 Intraoperative endoscopic evaluation through cervical esophagotomy, made just below the hypopharyngeal carcinoma.

One of the most difficult problems we confront clinically, in the preoperative examination, is the subgroup of patients where esophageal and gastric fiberscopy (GIF) could not be performed due to obstructive hypopharyngeal mass. Without knowning the existence of multiple lesions, we cannot decide the proper therapeutic (surgical) plan. Martins *et al*[9] reported that esophagoscopy and esophagography was attempted in 97% of patients in their series, but these examinations failed to evaluate the entire esophagus in 46% patients because of severe obstruction. In addition, very few patients (5 out of 36) underwent lugol staining during esophagoscopy. Although all patients were appropriately studied preoperatively, in only two cases multiple tumors were diagnosed before surgery. The remaining multiple synchronous carcinomas were obviously missed. Second primary tumor was, as a rule, located distal to the main obstructing carcinoma, preventing adequate total esophageal examination. Akyiama[16] cited intraoperative esophagoscopy as an important step in such patients. However, this procedure is not possible when tumors widely infiltrate cervical esophagus. Intraoperative endoscopy made through incision below the tumor, could be performed in most patients with obstructive carcinoma of postcricoid region, even when tumors infiltrate the esophageal ostium.

To justify the selective use of intraoperative endoscopy as a diagnostic tool for patients with obstructive hypopharyngeal carcinoma, a number of issues need to be considered. The procedure should significantly improve the diagnosis of synchronous primary tumors when compared with non-invasive radiologic investigations, including barium swallow (BaSw) and chest computed tomography (CT) or magnetic resonance imaging (MRI). Symptom directed studies are not feasible because patiens already have severe dysphagia due to obstructive proximal

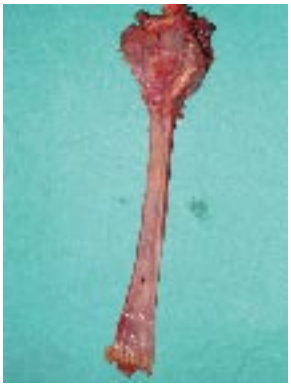

Figure 2 Synchronous invasive carcinomas of the hypopharynx and thoracic esophagus.

carcinoma. In addition, second primary tumor could be of early stage and does not cause subjective symptoms even in absence of proximal obstruction. Kohmura *et al*[6] support the view that in patients with obstructive hypopharyngeal carcinomas MRI should be performed. According to this data, esophageal mass lesions or hypertrophic mucosa detected by MRI require esophageal blunt dissection, due to possibility of multiple primary malignancies. In addition, Kohmura *et al*[6] proposed that there is little chance of multiple malignancies in the esophagus if there are no abnormalities by MRI. They also pointed out that endoscopic evaluation is favorable whenever is feasible. However, many authors agree that the role of MRI in the examination of gastrointestinal tract, apart from the liver, is limited[17,18]. CT has shown similar limitations in the diagnosis and staging of esophagogastric tumors with accuracy rate of only 60% or less[19,20]. Many others[21-23] agree that endoscopic ultrasonography (EUS) has clearly showed superior accuracy for T and N staging of esophageal and gastric carcinoma as compared with CT, but has the same limitations as endoscopic examination in the patients with obstructive tumors. Miniprobe sonography showed favourable results in the diagnosis and staging of esophageal tumors[18], but unfortunately, in most institutions is not available in routine clinical practice.

The second issue to be addressed is whether the diagnosis of a second primary tumor would change the primary treatment approach for the individual patient. Panosetti *et al*[24] in large series of patients, demonstrated that the discovery of a synchronous second primary tumor altered the treatment approach in 50% of patients. Many reports favour free jejunal interposition as a reconstructive method rather than gastric transposition[25-27]. These approaches may leave behind a premalignant or malignant lesion in the esophagus. One of the strongest arguments for total pharyngolaryngoesophagectomy and gastric transposition is the presence, or possibility of synchronous or metachronous primary in the esophagus. But then, stomach should be completely evaluated by endoscopy and x-ray before surgery, due to possibility of presence of the malignant tumor within stomach. Since the stomach is commonly used for reconstruction of the digestive tract after esophagectomy, another substitute must be considered for patients with synchronous gastric cancer.

The third issue to consider is the prognosis of patients with synchronous primary tumors, and impact of detection on overall survival. Advances in therapeutic methods have significantly improved local control rates. Still, many patients are developing distant metastases. Panosetti *et al*[24] reviewed the impact of survival of patients with synchronous versus metachronous second primary tumors. In a large series, these authors demonstrated that patients who were initially seen with synchronous primary tumors had 5-year survival rates of 18% *vs* 55% for those with metachronous tumors. These suggest that the overall survival for patients with detectable synchronous primary is quite poor. Martins *et al*[9] showed that more than 80% of second synchronous primaries were invasive carcinomas. In contrary, Kumagai[10] and Kohmura[6] found that most of the esophageal carcinomas accompaning advanced hypopharyngeal carcinomas were of early stage and that surgical excision could positively influence the prognosis. Thus, with the possibility of multiple intraesophageal cancer, endoscopic screening of the esophagus with lugol dye method in patients with head and neck cancer is necessary before treatment[13,28,29]. In our series, single synchronous primary was relatively small, but invasive (pT2) tumor of the thoracic esophagus, was visible without lugol staining (Figure 2).

Considering low incidence of obstructive hypopharyngeal carcinomas and high sensitivity of intraoperative endoscopy in detection of second primary, there is no need to consider whether this diagnostic procedure is cost-effective or not. More important is that there are no complications related to the procedure. One of the things that might be of concern is potential contamination of the operating field during the procedure. In our experience there were no local infective complications associated with the procedure. Therefore, benefit of the procedure exceeds potential risk of local contamination.

In summary, it is reasonable to use intraoperative endoscopy as selective screening test in patients with obstructive hypopharyngeal carcinoma.

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RAPID COMMUNICATION

Prognostic value of additional pathological variables for long-term survival after curative resection of rectal cancer

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Abstract

AIM: To evaluate the prognostic value of some pathological variables in rectal cancer survival.

METHODS: 247 patients who underwent curative resection of rectal cancer were included in the study. The influence on survival of five pathological variables (histopathological tumor type, histopathological tumor grade differentiation, blood vessel invasion, perineural invasion and lymphatic invasion) was assessed using statistical analyses.

RESULTS: Overall 5-year survival was 71.2%. Univariate analysis of all tested variables showed an effect on survival but only the effect of lymphatic invasion was statistically significant. At stages three and four it had a negative effect on survival (P = 0.0212). Lymphatic invasion also significantly affected cancer related survival in multivariate analysis at stages three and four. At lower stages (stage 0, stage 1 and stage 2) multivariate analysis showed a negative effect of perineural invasion on cancer related survival.

CONCLUSION: Patients with lymphatic and perineural invasion have a higher risk for rectal cancer related death after curative resection. Examination of these variables should be an important step in detecting patients with a poorer prognosis.

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Key words: Rectum; Cancer; Prognosis; Survival; Lymphatic invasion; Perineural invasion

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INTRODUCTION

Rectal cancer is often a curable disease and survival is directly correlated with the stage of the tumor, assessed by indicating the depth of penetration of the tumor into the bowel wall (T stage), the extent of lymph node involvement (N stage), and the presence of distant metastases (M stage). Besides the degree of penetration and presence of nodal involvement or distant metastasis, many other clinical, histological and biomolecular variables have been evaluated in the prognosis of patients with rectal cancer, although most have not been prospectively validated[1-3]. Among those, pathological factors are especially important as they may help to sub stratify tumors of same stage into different risk categories[4].

In Slovenia as in most countries, patients with radically resected rectal cancer with negative lymph nodes do not receive adjuvant systemic therapy, since significant survival benefits have not yet been proven[5]. One third of those patients, however, will develop local or distant metastases[6]. Identifying such patients would be very important because they could benefit from adjuvant systemic treatment.

The aim of our study was to retrospectively examine five histopathological prognostic factors which are routinely assessed and which may help to identify patients who could be included in an adjuvant chemotherapy program.

Histopathological tumor type (H)

The majority of colorectal cancers are adenocarcinomas. Some subtypes, like mucinous carcinoma (defined in the recent TNM classification as tumors with mucinous areas comprising more than 50% of tumor), signet ring cell carcinoma, and undifferentiated carcinoma, have a worse prognosis than adenocarcinoma.

Degree of differentiation (G)

In some multivariate studies, the microscopic grade is predictive of survival; a higher grade is known to be associated with a significantly increased risk of an adverse outcome.

Blood vessel invasion (BVI)

Blood vessel (venous) invasion by tumor has been demonstrated to be a stage-independent adverse

prognostic factor by many analyses[7-9]. However, some studies identifying venous invasion as an adverse factor on univariate analysis have failed to confirm its independent impact on prognosis in multivariate breakdowns[12].

Lymphatic invasion (LI)

Similar disparate results have also been reported for lymphatic invasion[10-11]. Therefore, data from existing studies are difficult to interpret. Nevertheless, the importance of venous and lymphatic invasion by the tumor is strongly suggested and largely confirmed by the literature[4].

Perineural invasion (PNI)

PNI may influence prognosis after resection of rectal cancer. An abundant extramural autonomic nerve network is an anatomical feature of the rectum. The evidence for the importance of perineural invasion is weaker than for blood vessel or lymphatic invasion but it has been shown in some series to be a predictor of both local failure and worse survival[13-15].

MATERIALS AND METHODS

Since 1998, data from all rectal cancer patients in our institution have been simultaneously registered in specially designed protocols, which contain preoperative, operative and postoperative parts and detailed pathological report.

The stage of the rectal cancer was defined according to the recent TNM classification[16].

Blood vessel invasion was considered positive if the following criteria were fulfilled: tumor cells at endothelial surface, tumor cell thrombi inside the lumen of the vessel or destruction of the vascular wall by tumor. Lymphatic invasion was defined as positive when tumor cells were found within endothelium-lined space devoid of mural smooth muscle or elastic fibers. Perineural invasion was considered positive when tumor cells were detected along, or around, a nerve within the perineural space.

Data from all patients who were operated on between January 1998 and December 2003 were analyzed. Over this period 512 patients with rectal carcinoma underwent surgery, but only 247 patients were eligible for the study, which included patients with elective radical resection of a solitary rectal tumor who survived the first month after the operation.

All patients were followed up on regular basis: at 3 mo intervals during the first 2 years, at 6 mo intervals until 5th year and then yearly. Clinical examination, tumor markers (CEA and CA19-9) and ultrasound were performed at each appointment. Chest X-ray was performed every 6 mo during first 2 years and then yearly. Colonoscopy was performed yearly after the operation. Follow up was updated on March 1st 2005.

We analyzed the following pathological variables: Histopathological tumor type, tumor grade or differentiation (well, moderately well, poorly differentiated and undifferentiated), blood vessel invasion, lymphatic and perineural invasion (present or absent).

Mortality data was acquired from the Slovenian cancer register and cause was verified for each deceased patient.

The research received approval from our ethics committee.

Survival was calculated using the Kaplan-Meier method and compared by log-rank test. Multivariate analysis was performed using Cox's regression model. All analyses were performed using statistical software SPSS for Windows 10.0.

Table 1 Basic demographic analysis

Age (yr)	65.8 (range 34-90)
	n (%)
Sex	
Men	138 (56)
Women	109 (44)
Follow up (d)	Median 1055 (range 31-2591)

Table 2 Distribution of patients according to their stage of disease

UICC	*n* = 247	%
0	2	1
1	54	22
2	116	47
3	72	29
4	3	1

RESULTS

The main operative procedures for rectal cancer in our institution are radical low anterior resection and abdominoperineal resection. Both procedures are executed according to principles of total mesorectal excision.

All patients were postoperatively managed by oncologists. The majority of patients with stage 3 and 4 disease received postoperative chemotherapy if there were no general contraindications.

The mean follow up time was 1055 days. The basic demographic analysis is shown in Table 1.

Most of the patients had stage 2 disease (47%), 29% had stage 3 and 22% stage 1. Only one percent of patients were at stage 0 and stage 4, as shown in Table 2.

5-year cancer related survival was calculated using Kaplan-Meyer method and was 71.4% for all stages.

For stage 1, survival was 91%, for stage 2.74%, for stage 3.51% and for stage 4. 33% (Figure 1).

For further analysis patients were divided into two groups according to stage. One group consisted of patients with stage zero, one and two tumors, and the second group consisted of patients with tumor stage three and four (Table 3).

The histopathological type of tumor and the degree of differentiation affected survival in lower and higher stages but this difference was not statistically significant.

Vascular and perineural invasion also showed no effect on 5-year survival in lower and higher stages.

Lymphatic invasion showed no effect on survival in lower stages but in higher stages, positive lymphatic invasion had a statistically significant negative effect on survival (log rank, $P = 0.0212$) (Figure 2).

Table 3 Univariate survival analysis of pathological variables according to stage

Variable	All Cases		LOWER STAGES (Stages 0, 1 and 2)		HIGHER STAGES (Stages 3 and 4)	
			5-yr cancer related survival (%)	*P* (log-rank test)	5-yr cancer related survival (%)	*P* (log-rank test)
	n	%				
Histological type	*n* = 246					
Adenocarcinoma	214	87	79	0.4056	42	0.5016
Mucinous carcinoma	23	9	83		74	
Other types	9	4	/		66	
Differentiation	*n* = 235					
G1	70	30	77	0.1312	43	0.5792
G2	141	60	87		63	
G3	21	8.9	51		42	
G4	3	0.1	/		/	
Blood vessel invasion	*n* = 226					
Positive	28	12	71	0.5920	46	0.2009
Negative	195	88	77		54	
Perineural invasion	*n* = 224					
Positive	31	14	74	0.1143	31	0.4247
Negative	193	86	78		53	
Lymphatic invasion	*n* = 218					
Positive	30	14	85	0.9083	27	0.0212
Negative	188	86	76		68	

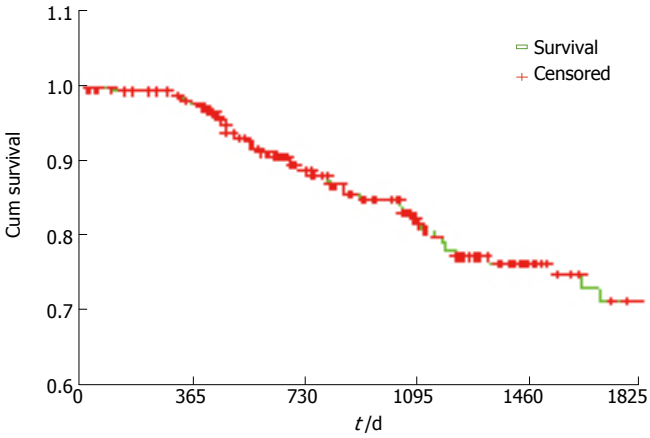

Figure 1 Cumulative 5-yr cancer related survival of all radically operated rectal cancer patients calculated with Kaplan-Meyer method.

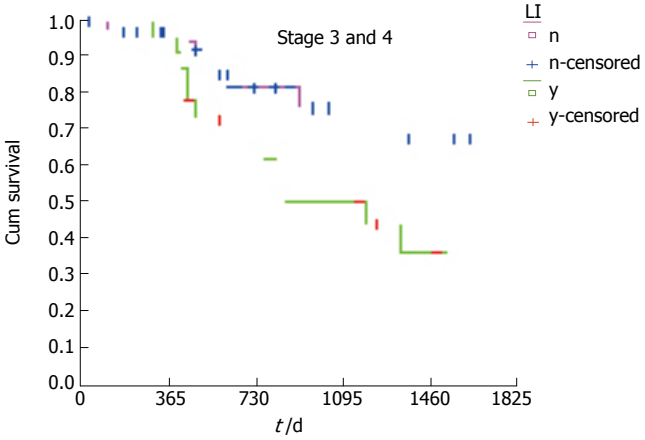

Figure 2 5-yr survival of radically operated rectal cancer (stage three and four) related to the presence or absence of lymphatic invasion. The difference is statistically significant ($P < 0.005$). Kaplan-Meyer method.

Multivariate analysis

Using Cox regression model on all previous variables we obtained different results for the different stages. Examining patients with higher stages we again confirmed that lymphatic invasion is an important prognostic variable. In multivariate analysis, lymphatic invasion clearly emerged as a strong negative prognostic factor ($P = 0.022$).

On the other hand, when patients with lover stages were considered, another variable, perineural invasion, seemed to have the greatest effect on survival ($P = 0.036$).

DISCUSSION

Rectal cancer is one of the leading causes of cancer-related deaths in the developed world[17].

Slovenia has one of the highest incidences of rectal cancer (men: 23.7 per 100 000, women 14.9 per 100 000). In 2001 there were 383 new cases of rectal cancer detected and 128 cancer-related deaths[18].

We know that more than two-thirds of patients with newly detected rectal cancer will undergo curative primary tumor resection and more than half will eventually die from the disease following surgery[19]. Survival is clearly dependent on rectal cancer stage but there are many other variables that could help us in predicting outcome. During recent years there were many studies and methods which focused on identification of factors that could influence postoperative survival after curative rectal cancer resection. These include serial sectioning, immunohistochemistry, and polymerase chain reaction assays. Examining all regional lymph nodes with these methods would be preferred, but is expensive and time consuming and therefore not feasible in daily practice.

Sentinel node-mapping offers a potential solution. It has been introduced in colorectal cancer to improve staging by facilitating occult tumor cell assessment in lymph nodes

that are most likely to be tumor-positive[20]. However, there is a large variation in identification rates and false-negative rates usually ascribed to the learning curve effect, differences in technique and tumor stage.

Compared to the methods described above, pathological variables, as assessed in this work, may have some advantages. They are not expensive and do not need any special resources.

In our study, lymphatic and perineural invasion were pathological variables that were significantly associated with pooreroutcomes in high and low stage groups, respectively. The importance of lymphatic invasion was already recognized in the late 1980's. Minsky found that it was an independent prognostic factor by proportional hazard analysis[21]. The importance of lymphatic invasion was confirmed in some later studies but also denied in others[22]. In our work it had a clearly negative effect on survival in a univariate analysis. In a multivariate analysis, the negative effect was seen only in patients with node positive rectal cancer. We assume that positive lymph nodes facilitate lymphatic invasion that could precipitate further spreading of the disease.

According to available data, perineural invasion may influence the prognosis after resection of rectal cancer[13]. In our work perineural invasion was associated with shorter survival in patients with lower stages of tumors. It is possible that the influence of positive perineural invasion in higher stages is masked by the importance of other parameters, and thus its role is clearly seen only in lower stages.

We must, however, mention some limitations of our study. First, although we report a 5-year overall survival, our median follow-up was 1055 d. However, we have used the Kaplan-Meier method to estimate 5-year survival. Second, we performed a retrospective study, and therefore our conclusions are limited by the bias inherent in an analysis of this nature.

In conclusion, additional pathological variables deservemore research to validate whether they will be helpful in predicting outcomes in rectal cancer patients. They are not expensive, are performed routinely and do not need any special resources. They could help with the identification of patients who would be predicted to have a poorer prognosis after curative resection of rectal cancer, who accordingly might benefit from adjuvant oncological treatment.

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RAPID COMMUNICATION

Time trends of incidence of digestive system cancers in changle of China during 1988-2002

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Abstract

AIM: To analyze the incidence of digestive system cancer in Changle of China over a 15-year period.

METHODS: The datasets were presented as time-series of China-standardized annual incidence during 1988-2002. Linear regression model was used to analyze the incidence of stomach, liver, esophagus and colorectal cancers.

RESULTS: Linear regression models for the time-series of stomach and esophagus cancer incidences for both men and women were statistically significant ($P < 0.05$); Regression models for liver cancer and for colorectal cancer were statistically significant for men ($P < 0.05$).

CONCLUSION: The incidence rates of stomach and esophagus cancers for both men and women had down tendencies. For men, liver cancer had a down trend of the incidence and colorectal cancer had an upward trend of the incidence rate.

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Key words: Stomach; Esophagus; Liver; Colorectum; Cancer; Incidence; Pattern; China

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INTRODUCTION

Cancer is the first cause of death in Fujian Province of China, and the proportion of cancer-related deaths is the highest. The mortalities of malignant digestive system tumors in Changle during 1988-1991 were the highest in Fujian Province and the incidence (per 100 000 persons) of stomach, liver, esophagus and colorectal cancers in Changle is 104.3%, 37.3%, 25.6% and 7.4%, respectively[1]. Since 1988, efforts have been made in prevention and treatment of tumors[2]. However, the patterns and incidence of malignant digestive system tumors in Changle are not available. Monitoring and studying the incidence of malignant tumors can provide important information for prevention and control of tumors. Therefore, the aims of the present study were to examine the pattern and time trends of stomach, liver, esophagus and colorectal cancers from 1988-2002 in Changle.

MATERIALS AND METHODS

Cancer incidence data

Annual stomach, liver, esophagus and colorectal cancer cases were provided by the Tumor Registration Office of Changle. Data of the age- and sex-specific population in Changle were provided by the local police station. The incidence of these tumors was standardized with the age- and sex-specific population obtained from the National Population Overall Survey in 1983. The datasets were presented as time-series of China standardized annual incidence during 1988-2002.

Statistical analysis

Linear regression model was used to analyze the incidence of stomach, liver, esophagus and colorectal cancer. The SAS software package was used for all analyses[3].

RESULTS

Standard incidence of digestive system cancer in men and women of Changle is shown in Table1. The incidence of stomach, liver and esophagus cancer in men decreased with fluctuation while that of colorectal cancer increased slowly from 1988 to 2002 (Figures 1A and 1B). On average, the incidence of stomach, liver and esophagus cancer in males decreased 3.44%, 3.16% and 6.07%, respectively. The incidence of these tumors except for stomach cancer in females was not significantly different (Figure 1C). The incidence of stomach, liver and esophagus cancer in women decreased 2.21%, 1.20% and 2.50%, respectively. The incidence of colorectal cancer in men increased 5.51% from 1998 to 2002.

Table 1 Standard incidence of digestive system cancer per 100 000 persons in 1988-2002 in Changle (%)

Yr	Stomach cancer		liver cancer		Esophagus cancer		Colorectal cancer	
	Male	Female	Male	Female	Male	Female	Male	Female
1988	106.63	28.54	32.62	10.48	20.22	6.36	3.1	6.3
1989	114.41	36.55	35.09	9.87	24.96	8.18	4.96	4.69
1990	109.09	29.59	51.86	10.76	28.81	12.48	5.93	4.56
1991	103.21	34.52	38.62	12.78	30.76	9.95	4.89	1.18
1992	100.81	26.1	32.47	7.66	25.52	7.45	2.44	4.11
1993	111.65	27.35	36.25	14.36	27.22	7.91	2.82	2.86
1994	98.73	24.9	44.54	11.09	22.38	7.92	4.04	2.16
1995	80.95	20.9	32.71	9.87	18.57	8.13	5.58	5.57
1996	85.42	19.52	32.87	7.63	18.7	4	6.24	4.43
1997	86.4	26.56	27.86	7.81	14.47	2.77	9.6	4.68
1998	81.81	21.82	35.48	16.29	12.96	4.07	5.86	5.33
1999	75.84	20.71	32.76	9.2	12.54	3.59	6.76	3.86
2000	83.96	25.63	32.81	10.99	15.15	2.63	5.39	3.82
2001	74.28	27.71	33.12	5.17	11.15	4.29	9.12	5.38
2002	65.33	20.88	20.81	8.85	8.41	4.46	6.57	8.03

Table 2 Incidence of four tumors both in men and women in Changle from 1988 to 2002 (%)

Disease	Men			Women		
	Regression coefficient	R^2	*P*	Regression coefficient	R^2	*P*
Stomach cancer	3.16	0.851	< 0.001	0.72	0.415	0.009
Liver cancer	0.83	0.286	0.04	0.16	0.061	0.374
Esophagus cancer	1.33	0.736	< 0.001	0.48	0.567	0.001
Colorectal cancer	0.29	0.391	0.013	0.12	0.1	0.251

The incidence of digestive system tumors both in men and in women was analyzed by linear regression models (Table 2). The regression coefficients for both men and women, except for colorectal cancer, were negative. The incidence of stomach and esophagus cancer in both men and women decreased ($P < 0.05$). The incidence of liver cancer was decreased in men ($P < 0.05$) but not in women. The incidence of colorectal cancer increased in men ($P < 0.05$) but not in women.

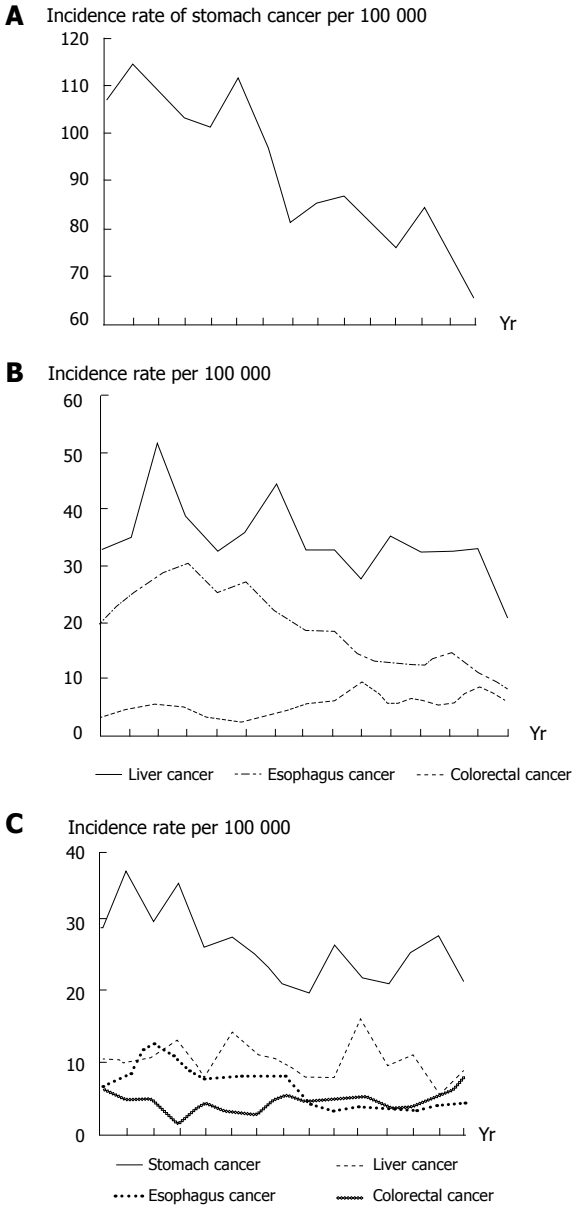

Figure 1 Incidence of stomach cancer (**A**) and liver, esophagus, colorectal cancer (**B**) in men, and four tumors (**C**) in women of Changle from 1988 to 2002.

DISCUSSION

This report presents the time-series analysis of standardized annual incidence of digestive system cancers in Changle City of China from 1988 to 2002. The incidence of stomach and esophagus cancers in men and women decreased 3.44% and 2.21%, respectively. The incidence of liver cancer decreased 3.16% in men but not in women. The incidence of colorectal cancer increased 5.51% in men but not in women.

The incidence of stomach and esophagus cancers in men and women decreased in Changle of China from 1988 to 2002, suggesting that the decreased incidence of these tumors is due to the development of economy and effect of tumor prevention in Changle. Epidemiological studies have shown that economic level and dietary habits are associated with the incidence of stomach and esophagus cancer[2,5-7]. Stomach cancer is the most common cancer in Japan and the Japanese have the highest mortality and incidence of stomach cancer in the world. The incidence and mortality of stomach cancer have declined in Japan[4,8]. The incidence of stomach cancer in a region is related to both economic level and dietary habits. Before 1980s, people in Changle of China had a low living standard and were accustomed to eating salted food and low protein and vitamin C diet. As the economical level is raised and knowledge of tumor prevention is spread in Changle, the people have changed their dietary habits which plays an important role in decreasing the incidence of stomach and esophagus cancer. During 1988-2002, stomach cancer incidence decreased about 38.73% in males and 26.84% in females, and esophagus cancer incidence decreased about 58.41% in males and 29.87% in females in Changle of China. However, stomach cancer is still the most

common cancer in Changle and its incidence in males is still the highest in China although the disease incidence is declined. Therefore, research on preventive factors, such as eating habits, foods, additives, drug use, prevalence of Helicobacter pylori infection and environment pollution, is needed.

Our study showed that the digestive system cancer incidence was different in Changle. The incidence of stomach and esophagus cancer declined while colorectal cancer incidence increased in Changle, which is in accordance with the incidence in Japan[11]. Colorectal cancer incidence tends to increase in males of Changle, and the incidence of the disease in males in 2002 was 2.12 times higher than that in 1988. Colorectal cancer is common in many developed countries[12,13]. Countries where the people have a high fat intake also have high colorectal cancer incidence[14]. High fat intake is a risk factor for colon polyp which may progress to colorectal cancer[15], and 15%-25% of colorectal cancer cases may be attributable to high fat intake[16]. Moreover, high vegetable and fruit consumption and less meat consumption are associated with a reduced incidence of colorectal cancer[17-19]. Therefore, a variety of dietary interventions may have a positive impact on dietary behaviors associated with cancer risk[20].

During 1988-2002, the incidence of liver cancer in males and females were 34.66% and 10.19% in Changle, respectively. It was reported that the incidence of liver cancer is decreased[21] and liver cancer incidence is increased in China[22, 23].

The incidence of digestive system cancer is different in different areas of China. The reasons for the declined incidence remain unclear. Elimination of HBV-cofactors is likely to contribute to it. However, some risk factors for liver cancer, including smoking and alcohol consumption, are still highly prevalent in males in Changle of China. Therefore, our results might be affected by the lowest incidence in 2002. To confirm whether liver cancer incidence is decreased, further observations are required.

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RAPID COMMUNICATION

Preventive effects of chitosan on peritoneal adhesion in rats

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Abstract

AIM: To study the effects of chitosan gel and blending chiston/gelatin film on preventing peritoneal adhesion in rats.

METHODS: SD rats were randomly divided into 2 groups, group A treated with chitosan gel and group B with blending chiston/gelatin film. In group A, rats were randomly subdivided into 3 subgroups as groups A1, A2 and A3, and different methods were used to induce peritoneal adhesions at the dead end of vermiform process in each group as follows: Group A1 with trauma, A2 with talc powder and A3 with ligation of blood vessel. In each subgroup, rats were redivided into control group and experimental group whose treated vermiform processes were respectively coated with chitosan gel and normal saline immediately after the adhesion-induced treatments. In group B, all the rats received traumatic adhesion-induced treatments and then were randomly divided into 4 groups (groups B1, B2, B3, B4). Group B1 served as control group and were coated with normal saline in the vermiform processes immediately after the treatments, and groups B2, B3 and B4 with 100% chitosan film, chitosan film containing 10% gelatin and chiston film containing 50% gelatin, respectively. At 2 and 4 wk after the above treatments, half of the rats in each terminal group were belly opened, and the peritoneal adhesive situation was graded and histopathological changes were examined.

RESULTS: (1) In group A, regarding peritoneal adhesion situation: At both 2 and 4 wk after the treatments, for groups A1 and A3, the adhesive grades of experimental groups were significantly lower than those of the control group (2 wk: $H = 4.305$, $P < 0.05$ for A1, $H = 6.743$, $P < 0.01$ for A3; 4 wk: $H = 4.459$, $P < 0.05$ for A1, $H = 4.493$, $P < 0.05$ for A3). However, of group A2, there was no significant difference between the experimental and control groups (2 wk: $H = 0.147$, $P > 0.05$; 4 wk: $H = 1.240$, $P > 0.05$). Regarding pathological changes: In groups A1 and A3, the main pathological change was fibroplasia. In group A2, the main changes were massive foreign-body giant cell reaction and granuloma formation with fibroplasia of different degrees. (2) In group B, regarding degradation of film: With increase of the blended gelatin concentration, degrading speed of the film accelerated significantly. Regarding peritoneal adhesion situation: At both 2 and 4 wk after the treatments, the adhesive grades of B1 were the lowest among the four subgroups of B (2 wk: $H = 29.679$, $P < 0.05$; 4 wk: $H = 18.791$, $P < 0.05$). At 2 wk after the treatments, the grades of group B2 were significantly lower than that of groups B3 and B4 ($H = 4.025$, $P < 0.05$ for B2 *vs* B3; $H = 4.361$, $P < 0.05$ for B2 *vs* B4). At 4 wk, there were no significant differences of the grades between groups B2, B3 and B4. Regarding pathological changes: Inflammatory cell infiltration and fibroplastic proliferation were observed in the local treated serous membranes, which was the mildest in group B1. Slight foreign-body giant cell reactions were also found in groups B2, B3, and B4.

CONCLUSION: (1) Chitosan gel has preventive effect on traumatic or ischemic peritoneal adhesion, but no obvious effect on foreign body-induced peritoneal adhesion. (2) Chitosan film may exacerbate the peritoneal adhesion. Blending with gelatin to chitosan film can accelerate the degradation of the film, but can simultaneously facilitate the formation of peritoneal adhesion.

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Key words: Chitosan; Gelatin; Peritoneal adhesion; Rat

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INTRODUCTION

Chitosan is the derivant of the chitin after deacetylation, and chitin is a main ingredient of the arthropod shells (such as shrimps, crabs and insects, *etc*), which is a kind of renewable natural resources and profuse in amount.

Since chitosan is innocuous, biodegradable and with ideal biocompatibility, it has been applied to develop biomaterials[1,2]. In medical field, chitosan has antiseptic function and can facilitate the healing of wound. It has been widely studied on its potential use to be medical biomaterials. Because of its inhibitory effect on fibroblast growth and the function of mechanical isolation[3], chitosan has been regarded highly in the prevention of peritoneal adhesion. To evaluate the effects of different types of chitosan on preventing traumatic peritoneal adhesion, a control study in rats was done in this paper.

MATERIALS AND METHODS

Experimental animals and grouping

Two hundred and forty SD rats, 120 females and 120 males, weighing 200-250 g, were provided by the Laboratory Animal Center of Zhejiang Province. They were randomly divided into group A (144 rats) and group B (96 rats). Group A was treated with chitosan gel and group B with blending chiston/gelatin film. In Group A, 144 rats were randomly divided into 3 subgroups (groups A1, A2, A3) with 48 rats in each. Different methods were used to induce peritoneal adhesion in the dead ends of the vermiform processes as follows: group A1 with trauma, group A2 with talc powder and group A3 with ligation of blood vessel. For each subgroup, 48 rats were randomly redivided into control group and experimental group with 24 rats in each. In group B, all the 96 rats received traumatic adhesion-induced treatments as group A1 and then were randomly divided into 4 groups (groups B1, B2, B3, B4) with 24 rats for each. Group B1 served as control group and groups B2, B3, and B4 served as experimental groups treated with 100% chitosan film, chitosan film containing 10% gelatin and chiston film containing 50% gelatin, respectively.

All the rats were fed under the same condition: 24℃ −26℃ of environmental temperature, about 40% of humidity, alternating 12 h light/dark cycle, free access to food and water.

Surgical methods: Under general anaesthesia with intraperitoneal injection of 3% amobarbital (60 mg/kg), the rats were immobilised in dorsal position, routinely degermed, abdominally incised through a median incision of 2-3 cm long, and the vermiform processes were searched and pulled out of the incision, then the terminal vermiform processes within a distance of 3 cm were treated as follows: In group A1, the anterior surface of serous membrane was scraped slightly with surgical blade till obvious congestion and small bleeding drops appeared. In group A2, talc powders were evenly smeared over the anterior surface of serous membrane. In group A3, the vermiform artery stem was ligated with No 0 surgical thread at the point of 3 cm from the dead end in the following way: loosely knotting the first loop, thrilling a thread with equivalent diameter to the vermiform artery stem through the first loop, tightening the first loop, knotting and tightening the second loop of the ligation knot,and pulling out the thrilled thread. The ligation resulted in a stricture of vermiform artery which induced ischemia of the distal vermiform tissue from the ligation point. This method had been proved successful in inducing peritoneal adhesion in our former experiments. After the above treatments, for the experimental groups, the treated serous membranes within 3 cm distance from the dead ends were coated with chitosan gel in a dosage of 0.5 mL for each, and the vermiform processes were put back into the abdominal cavities, which were then closed. For the control groups, all the treatments were the same as those of the experimental groups except that the chitosan gel was replaced by normal saline. The duration from opening to closing the abdominal cavity was 5 min, so that the duration of exposure of intestines to air was the same for each rat. In group B, of all the rats, the anterior surfaces of vermiform processes were scraped slightly with surgical blade just as group A1, and the scraped surfaces of B2, B3, B4 were coated with films containing different percentages of gelatin at 0%, 10%, and 50% respectively. Group B1 was treated with normal saline as control. The duration from opening to closing the abdominal cavity was controlled just for 5 min.

At 2 and 4 wk after the surgery, 12 rats (6 females and 6 males) in each subgroup were randomly selected respectively for further study. The abdominal cavity was reopened under anaesthesia, and the grades of peritoneal adhesion were evaluated which existed between the treated vermiform processes and intestines, mesenteria and abdominal walls. After that, the vermiform processes with adhesions were resected, fixed with formalin and histopathologically examined.

Grading standard for the peritoneal adhesion

Referring to Phillips'[4] grading method of 5 levels and considering the characteristics of peritoneal adhesion in rats, we offered the following grading standard: Grade 0: no adhesions; Grade Ⅰ: the ratio of adhesive area/the total treated area in the vermiform processes < 20%; Grade Ⅱ: the ratio is about 40% Grade Ⅲ: the ratio is about 60%; Grade Ⅳ: the ratio is ≥ 60%. Each rat was graded by three referees blindly and the average grade of the three was accepted as the adhesive grade of the rat.

Statistical analysis

The H-test of non-parametric statistics for ranked grouped data was used to analyze the differences of the peritoneal adhesive grades between the experimental and control groups. $P < 0.05$ was taken as significant.

RESULTS

Group A

Observation with naked eyes: The skin incisions of all rats healed in first grade. No obvious infection appeared in the abdominal cavity in all rats. In the experimental groups, no obvious residual of chitosan gel could be found at 2 wk after surgery.

Comparison of peritoneal adhesion level: As it shows in Table 1, in groups A1 (trauma-induced adhesion) and A3 (ischemia-induced adhesion), peritoneal adhesion grades of the experimental groups were significantly lower than those of the control groups ($P < 0.05$ or $P < 0.01$) both at 2 and 4 wk after the surgical treatments. While in

Table 1 Comparison of peritoneal adhesion between experimental and control groups in groups A1, A2, and A3

Group		Experimental group (total n = 12)					Control group (total n = 12)					H	P
		0	I	II	III	IV	0	I	II	III	IV		
A1	2 wk	1	7	4	0	0	5	6	1	0	0	4.305	< 0.05
	4 wk	2	7	3	0	0	6	6	0	0	0	4.459	< 0.05
A2	2 wk	0	1	1	4	6	0	0	2	3	7	0.147	> 0.05
	4 wk	0	0	1	2	9	0	0	0	1	11	1.240	> 0.05
A3	2 wk	0	5	5	2	0	2	8	2	0	0	6.743	< 0.01
	4 wk	2	5	4	1	0	6	5	1	0	0	4.493	< 0.05

Table 2 Comparison of peritoneal adhesion in group B rats

Group	n	2 wk (n)					4 wk (n)				
		0	I	II	III	IV	0	I	II	III	IV
B1	24	1	7	4	0	0	2	7	3	0	0
B2	24	1	3	3	4	1	0	3	3	5	1
B3	24	0	0	2	4	6	0	2	2	3	5
B4	24	0	0	0	2	10	0	2	1	5	4
H		29.679					18.791				
P		< 0.05					< 0.05				

group A2 (talc powder-induced adhesion), there was no significant difference in the peritoneal adhesion between the experimental and control groups ($P > 0.05$) both at 2 and 4 wk. The above results indicated that chitosan gel has perfect effect on preventing peritoneal adhesion induced by trauma and ischemia, but no obvious effect on adhesion induced by talc powder.

Comparison of pathological changes: In group A1, at 2 wk after the surgical treatments, there existed obvious fibroplasia and diffused lymphocytes infiltration in the treated serous membrane of the vermiform processes. While at 4 wk after the treatments, the main pathological change was fibroplasia. The above pathological changes in the experimental group were obviously milder than those in the control group. In group A2, at 2 wk, massive foreign-body giant cell reaction (FBGCR) and granuloma formation appeared in the treated vermiform processes with diffuse inflammatory cell infiltration. At 4 wk after the surgical treatment, foreign body granuloma and fibroplasia reaction aggravated with crystal appearing in the foreign-body giant cell. There was no significant difference in the adhesive severity between the experimental and the control groups. In group A3, 2 wk after the surgical treatments, full-thickness fibroplasias and lymphocyte infiltration appeared in the vermiform processes which was more severe in the serous layer. While at 4 wk after the treatment, the lymphocyte infiltration became lighter, but the fibroplasias aggravated obviously. The above pathological changes in the experimental group were obviously milder than that in the control group 2 wk after the treatment. However, at 4 wk, there was no significant difference between the experimental and control groups.

Group B

Observation with naked eyes: The skin incisions of all rats healed in first grade. No obvious infection appeared in the abdominal cavities at 2 wk after the treatments in all rats. Chitosan film degradation: In group B2, no obvious changes occurred in the chitosan film at 2 wk, and even no obvious rupture could be found in the film at 4 wk after the surgical treatments. The films were only swollen and thinned at 4 wk. In group B3, the films with 10% gelatin were swollen at 2 wk, and degradated into fragments at 4 wk after the treatments. In group B4, the films with 50% gelatin were broken into pieces at 2 wk, and disappeared at 4 wk after the surgical treatments.

Comparison of peritoneal adhesion degree: As listed in Table 2, both at 2 wk and 4 wk after the surgical treatments, there existed significant differences among the four groups ($P < 0.05$) in the peritoneal adhesive degree. At 2 wk, all the differences were significant between every 2 groups ($P < 0.05$) except between groups B3 and B4. At 4 wk, there were significant differences between groups B1 and B2, B1 and B3, B1 and B4 ($P < 0.05$), but there was no significant difference between groups B2 and B3, B2 and B4, B3 and B4 ($P > 0.05$). All the above results indicated that both pure chitosan film and blending chiston/gelatin films could exacerbate peritoneal adhesion, as well as the blended gelatin.

Comparison of pathological changes: (1) At 2 wk after the surgical treatments, the adhesive vermiform processes of all rats were mainly with various degrees of edema, dilation and congestion of capillaries, infiltration of inflammatory cell and fibroplasias. The reaction became severer in the serous membrane than in other layers. The reactions in groups B2, B3, and B4 were more severe than those in group B1. There were slight foreign-body giant cell reactiond in groups B2, B3 and B4 (Figure 1). (2)At 4 wk after the treatments, the acute inflammatory reaction decreased obviously in all groups except group B1. In all the other groups (B2, B3 and B4), the fibroplasia was aggravated obviously which caused formation of complete fibrous capsules around the implantation, with a thickest capsule wall in group B4 and a thinnest one in group B2. Furthermore, groups B2, B3, and B4 had obvious foreign-body giant cell reaction (Figure 2).

DISCUSSION

Peritoneal adhesion is an inevitable phenomenon of the natural repairing processes after peritoneal injury. In recent years, many researches have been done on the process and mechanism of peritoneal adhesion. To understand the

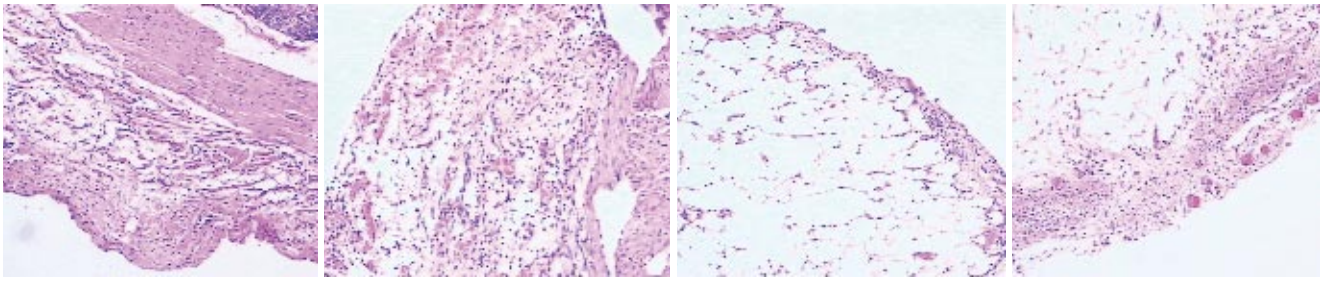

Figure 1 Histopathologic changes of the four B-groups at 2 wk after the surgical treatment.

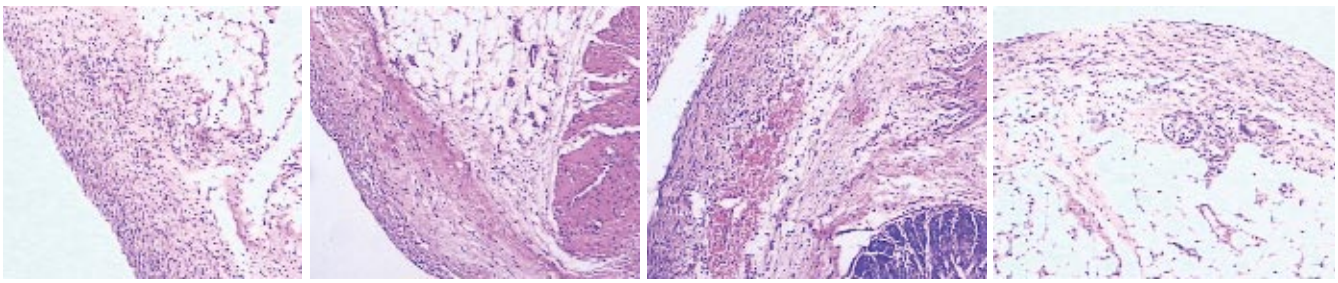

Figure 2 Histopathologic changes of the four B-groups at 4 wk after the surgical treatment.

mechanism of peritoneal adhesion is very important for the prevention of its formation.

Animal studies indicated that the serous membrane injury caused by mechanical injury, ischemia of tissue, stimulation of foreign body and peritonitis was the main reason of peritoneal adhesion. However, whether it results in permanent fibrinous adhesions or not depends on the integrity of the fibrinolytic system[5,6]. Generally speaking, the formation of peritoneal adhesion needs the following steps: step 1 is the formation of fibrinous gelatinous matrix. This happens within 3 h after the injury. The gelatinous matrix locates among the injured peritoneal membranes, which is the initiation of peritoneal adhesion. Step 2 occurs between 1-5 d after the injury. The fibrinous matrix is gradually replaced by the vascular granular tissues which contain fibroblasts, macrophagocytes and giant cells. Most of the fibrins disappear and are replaced by a large number of fibroblasts and collagen fibers, and then the fibrin network is formed. Step 3 takes place during 5-10 d after the injury. The fibroblasts acquire a regular alignment gradually and the collagen deposition is enriched. Step 4 is at 2 wk after the injury. The component cells, especially the fibroblasts, decrease obviously and are covered by mesothelial cells. Eventually, the fibrinous adhesions are formed. However, after peritoneal injury, the fibrin can be decomposed by fibrinolysin into fibrin degradation products (FDPs) in the course of fibrinous gelatinous matrix formation, which is unrelated with peritoneal adhesion. It generally occurs at 72-76 h after the injury. In brief, when peritoneal injury occurs, whether the wounds heal through adhesive fusion or through epithelization, mainly depends on the degree of local fibrinolysis and whether epilesional juxtaposition exists[7].

Based on how and why the peritoneal adhesion happens, more than 10 preventive methods have been proposed. Among them, mechanical isolation seems to have the brightest prospect. At present, close attention has been paid to the following 4 materials which are used as mechanical isolation: EPTEE, oxidized regenerated cellulose (ORC), HA-CMC and chitosan[8,9]. Chitosan is highly regarded as a biomaterial for prevention of peritoneal adhesion, because it has the functions of anti-infection, hemostasis, inhibiting growth of fibroblasts, mechanical isolation and moreover, it is biodegradable.

Chitosan is the derivant of deacetylized chitin. Chitin was discovered in 1811. Its molecular structure was determined by chemical method and X-ray diffraction in 1887. Chitin can be converted into chitosan after deacetylation. Chitosan is soluble, easily to be chemically modified because it contains many amid and hydroxyl. Meanwhile, this kind of natural polysaccharose is alkaline and has good biocompatibility and is biologically degradable. Its degradation products are acetylglucosamine and aminoglucose, which are atoxic for human bodies. The low molecular weight of chitosan and its oligosaccharide produced during degradation render it to have no immunogenicity and not to accumulate *in vivo*. The biological activity of chitosan mainly includes: (1) inhibiting the growth of bacteria and mold; (2) antineoplasmic activity: It can selectively agglutinate the L1210 cells in leukemia and Ehrlich's cells in carcinomatous ascites, but does not affect the normal erythroid bone marrow cells; (3) immunological enhancement: It can efficaciously enhance the function of macrophages and the activity of hydrolase, stimulate the macrophage to produce lymphokine and initiate the immune response. But it cannot promote the production

of antibody; (4) anticoagulant activity: The chitosan after thioesterification has a similar chemical structure with heparin so that it has a good anticoagulant activity; (5) promoting tissue repair and hemostasis: Since the degradating product of chitosan is charged, it can induce platelet aggregation and activate the coagulation system. Chitosan can inhibit fibrous hyperplasia during wound healing and efficaciously enhance wound healing. Animal experiments in recent years have proven that chitosan gel has good effect in preventing peritoneal adhesion[3,4].

All the 3 different animal models of peritoneal adhesion used in our study were monofactor-induced peritoneal adhesion, which made the study of effects of chitosan on preventing peritoneal adhesion due to different causes technologically possible. Observation at 2 and 4 wk after the surgical treatments in all the control groups showed that the incidence of peritoneal adhesion was 90.9% and 83.3% respectively, and most of the adhesions were graded as Ⅰ and Ⅱ. This proved that the animal model was reliable and stable.

In our study, different chitosan materials were used for the prevention of peritoneal adhesion, which turned out to have completely different results. Because the peritoneal adhesions in each group were caused by different methods, different preventing effect was observed. In group A (treated with chitosan gel), the chitosan gel film had satisfactory effects on the prevention of peritoneal adhesion due to injury or ischemia. At 2 wk after the treatments, the chitosan gel was completely biologically degraded. Moreover, the peritoneal adhesion level and pathological change in both groups A1 and A3 were milder than those in the control groups. This indicated that the chitosan gel had an evident preventing effect against peritoneal adhesion due to injury or ischemia. In group A2 (adhesions induced by talc powder), both histopathologic examination and adhesion grades indicated that the chitosan gel had no evident preventing effects on peritoneal adhesion induced by talc powder. The probable cause may be related with the pathological change in adhesions induced by talc powder, which mainly was foreign body granuloma reaction with massive fibroplasias. As long as the foreign body existed, the foreign body granuloma and fibroplasia reaction would remain persistent. Since the chitosan gel could be fastly degradated *in vivo* (within 2 wk after surgery), the acting time of the chitosan gel was too short to prevent against persistent foreign body granuloma reaction.

Chitosan gel is still imperfect because it is highly flowable. When chitosan gel is smeared at the surface of wounded peritoneum, it cannot come to a high concentration in the focus, resulting in weakened effect since its acting time is shortened. In order to improve the concentration of chitosan in the intraabdominal focus and make a more thoroughly mechanical isolation, some researchers used pure chitosan film instead to get a better effect on peritoneal adhesion prevention. However, our study indicated that such effort was disappointing.

In Group B2 (treated with pure chitosan film), at 4 wk after the treatments, the chitosan film was still undegraded. Such slow degradating speed was detrimental for an anti-peritoneal adhesion material because if the postsurgical initial membranous adhesions cannot be degraded in time, it will form irreversible fibrinous adhesions, which cannot be inhibited by chitosan. On the contrary, the intraabdominal residual of undegraded chitosan film can evoke the foreign body reaction and result in fibrous capsule formation, which facilitates the formation of peritoneal adhesion. These were proved in our study: the adhesion grade in group B2 was higher than that in control group, and histopathologic examination indicated obvious foreign-body giant cell reaction at 2 and 4 wk after the surgical treatments.

Because the slow degradation of chitosan film weakened its anti-adhesive function, blending chiston/gelatin film was suggested to improve the *in vivo* degradation speed of chiston film. And experiments proved that the more gelatin added in the film, the more fastly the blending film *in vivo* degradated. However, there have been no experimental data about the effect of the blending chiston/gelatin film on the prevention of peritoneal adhesion.

Gelatin is a polypeptide mixture which is soluble in hot water. It is widely used in medical field as haemostat and dermagraft and dressing materials of wounds because it can be degradated quickly *in vivo*. In our study, the blending chiston/gelatin films with different concentration of gelatin were used in group B2 (10% gelatin) and B4 (50% gelatin), and the results indicated that the higher concentration of gelatin in the film, the more fastly it was degradated in the abdominal cavity of rats. However, our study indicated that the anti-peritoneal adhesion effect of the blending film was not ideal. The peritoneal adhesion grade was not only lower than that of the control group, but also lower than that of group B2 (with pure chiston film). The probable reasons are: although the blending chiston/gelatin film had a faster degradation speed, the film still could not be completely degradated even at 4 wk after the treatments in group B3 and at 2 wk in group B2. The residual blending film may cause foreign body reaction. In addition, gelatin is a polypeptide mixture which is probably antigenic and can cause immunological rejection. This in turn promotes and facilitates the formation of local peritoneal adhesions.

We conclude that: (1) Chitosan gel has perfect effect on preventing peritoneal adhesion due to injury or ischemia, but no evident effect on peritoneal adhesion induced by talc powder; (2) Pure chitosan film could not prevent peritoneal adhesion because of low *in vivo* degradation speed; (3) Chitosan film blended with gelatin could exacerbate peritoneal adhesion.

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RAPID COMMUNICATION

Anti-inflammatory effect of Diammonium Glycyrrhizinate in a rat model of ulcerative colitis

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Abstract

AIM: To explore the anti-inflammatory mechanism of Diammonium Glycyrrhizinate in a rat model of ulcerative colitis induced by acetic acid.

METHODS: Spragur-Dawley female rats were divided into four groups: Diammonium Glycyrrhizinate group, dexamethasone group, acetic acid control and normal control group. Colonic inflammation was evaluated by disease activity index, gross morphologic damage, histological injury and colonic myeloperoxidase activity. Immunohistochemistry was used to detect the expression of NF-κB, TNF-α and ICAM-1 in colonic mucosa.

RESULTS: Compared to the acetic acid control, both Diammonium Glycyrrhizinate and dexamethasone showed a significant anti-inflammatory effect ($P < 0.01$). The expression of NF-κB, TNF-α and ICAM-1 in colonic mucosa was significantly lower in the Diammonium Glycyrrhizinate group and dexamethasone group than in the acetic acid group.

CONCLUSION: Diammonium Glycyrrhizinate could reduce inflammatory injury in a rat model of ulcerative colitis. This may occur via suppression of NF-κB, TNF-α and ICAM-1 in colonic mucosa.

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Key words: Ulcerative colitis; Diammonium Glycyrrhizinate; Mechanism

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INTRODUCTION

Traditional drugs for ulcerative colitis, though quite effective, may exert severe side-effects. New therapeutic agents with less side-effects would be very useful. Diammonium Glycyrrhizinate (DG) is a substance that is extracted and purified from a traditional Chinese medicinal herb. The herb has been used traditionally for the treatment of hepatitis due to its anti-inflammatory effect, resistance to biologic oxidation, membranous protection and a weak steroidal action. However, it was not known whether DG might also be effective in other inflammatory conditions, such as ulcerative colitis. To explore a possible anti-inflammatory effect of DG in ulcerative colitis, the expression of NF-κB, TNF-α and ICAM-1 in colonic mucosa was detected by immunohistochemistry in a rat model.

MATERIALS AND METHODS

Materials

Female SD rats were purchased from the Center of Experimental Animals in the 89th Hospital of PLA. NF-κB, TNF-α and ICAM-1 antibodies and immunohistochemistry kits were purchased from Beijing Zhongshan Biotech Company. MPO kit was purchased from the Institute of Nanjing Jiancheng Biotechnology. DG was purchased from Jiangsu Chia Tai Tianqing Pharmaceutical Co., LTD (X20010756, DG0267).

Methods

Preparation of animal model: Forty SD rats were divided into four groups: DG group, dexamethasone group, acetic acid control and normal control group. All rats were fasted for twenty-four hour. Before the colonic infusion of acetic acid, 0.3 mL (30 mg/kg) Natrium pentobarbital was injected peritoneally. A polyethylene catheter was put into the colon extending a distance of eight centimeters beyond the anus. For DG, dexamethasone, and the acetic acid control groups, 1 mL of 10% (v/v) acetic acid was

Table 1 Morphologic injury scoring system

Morphologic injuries	Score
Local edema and congestion without ulcer	1
One ulcer without congestion or thick colonic wall	2
One ulcer with inflammation	3
Two ulcers, lesion diameter less than 1 cm	4
More than two ulcers with or without inflammation, lesion diameter 1-2 cm	5
Lesion diameter over 2 cm, one more score for one additional 1 cm increasing diameter	6-8

Table 2 Histological scoring system

Histological changes	Score
Normal colonic mucosa	0
Cryptal defect less than 1/3	1
Cryptal defect 1/3-2/3	2
Slight inflammatory infiltration in proper lamina	3
Mucosal erosion or ulcer with significant inflammatory infiltration	4

infused into the colon through this catheter[1], held in place for 30 s, and then flushed with 5 mL normal saline. Only normal saline was infused into the colon in the normal control group. In the DG group, 40 mg/kg DG was injected intraabdominally every day for one week; in dexamethasone group, 0.2 mg/kg dexamethasone was injected intraabdominally cavity daily for one week; in the acetic acid control and normal control groups, equal volumes of normal saline were injected into the abdominal cavity daily for one week.

Preparation of colonic tissue samples: Seven days after acetic acid infusion, the body weight of each rat was measured and recorded. The distal colon was removed at the level of 8 cm from the anus. Samples were evaluated by morphological scores, and then cut into two parts-one for H&E staining and immunohistochemistry, the other for the detection of MPO activity.

Evaluation of colonic inflammation: Colonic inflammation was evaluated seven days after acetic acid infusion by disease activity index (DAI), morphologic injury, histological changes, and MPO activity. DAI was calculated as the sum of scores assigned as follows: percentage of body weight reduction (0: no change, 1: 1%-5%, 2: 6%-10%, 3: 11%-15%, 4: > 15%), Stool consistency (0: normal, 2: loose, 4: diarrhea) and the presence of fecal blood (0: normal, 2: positive occult blood test, 4: visible bleeding)[2]. Morphologic injury was evaluated using the scoring system reported by Dieleman, with slight modifications[3] (Table 1). Ten fields were selected randomly to estimate histological injury according to the scoring system listed in Table 2[4]. A kit was used to detect MPO activity in the colonic mucosa.

Immunohistochemical detection of NF-κB p65, TNF-α and ICAM-1: The expression of NF-κB p65, TNF-α and ICAM-1 in colonic mucosa was detected by a SP immunohistochemistry kit. On each slide, five fields (× 400) were selected randomly and the percentage of positive cells was counted among 1000 cells. The average of positive cells in five fields was the determined.

Statistical analysis

Data are presented as mean ± SD, and statistical differences among groups were tested using the SPSS statistical software package. Based on the result of a test of homogeneity of variances, one-way ANOVA and SNK-q test were applied to test differences between different groups. A *P* value of 0.05 was set as the level of statistical significance.

Table 3 DAI, morphologic injury, histological changes, and MPO activity in rats assigned to the different treatment groups

Group	DAI	Morphologic injury	Histological changes	MPO activity (U/g tissue)
DG	3.10 ± 0.54[b,d,f]	2.80 ± 0.76[b,d,f]	3.22 ± 0.88[b,d,f]	0.67 ± 0.06[b,d,f]
Dexamethasone	1.90 ± 0.43[b,d]	1.70 ± 0.54[b,d]	1.76 ± 0.59[b,d]	0.38 ± 0.05[b,d]
Acetic acid control	6.70 ± 0.84[b]	6.30 ± 0.95[b]	5.96 ± 0.97[b]	1.32 ± 0.09[b]
Normal control	0.60 ± 0.38	0.60 ± 0.52	0.74 ± 0.56	0.19 ± 0.05

[b]$P < 0.01$, *vs* Normal control group; [d]$P < 0.01$, *vs* Acetic acid control group; [f]$P < 0.01$, *vs* Dexamethasone group.

RESULTS

DAI, morphologic injury, histological changes, and MPO activity

1-2 d after colonic infusion of acetic acid, rats displayed diarrhea, pyemic stool, and reduced body weight. Morphologically, a dilated lumen, thickened wall, and brown or black color was observed continuously in the injured bowel. Edema, erosions, necrosis, superficial ulcerations, crypt abscesses, and inflammatory infiltration into the lamina propria were observed in the injured segment by light microscopy. In Table 3, according to DAI, scores of morphological and histological changes, and MPO activity, the colon showed significant pathogenic changes in the DG, dexamethasone, and acetic acid control groups compared to the normal control group that received saline alone ($P < 0.01$), which demonstrated that acetic acid infusion results in injuries that are comparable to those seen in humans with ulcerative colitis. These inflammatory indices were significantly improved by DG and dexamethasone ($P < 0.01$). The anti-inflammatory effect of DG was significantly lower than that of dexamethasone ($P < 0.01$).

Expression of NF-κB p65, TNF-α and ICAM-1 in injured colon

In rats that received acetic acid, NF-κB p65 was positive mainly in nuclei of most endothelial cells, epithelial cells and mononuclear cells, especially in the mucosa and submucosa. TNF-α and ICAM-1 were positive mainly in the cytoplasm, membrane and rarely in nuclei. ICAM-1 was positive in most endothelial cells and macrophages. TNF-α positive cells, including mononuclear cells, macrophages and neutrophils, were located densely in lamina propria and in proximity to the muscularis. The percentage of cells positive for these three molecules was significantly correla-

Table 4 Expression of TNF-α ,ICAM-1 and NF-κB p65 in injured colon

Group	TNF-α		ICAM-1		NF-κB p65	
	Percentage	Density	Percentage	Density	Percentage	Density
DG	32.2 ± 8.2[b,d,f]	37.3 ± 7.0[b,d,f]	34.3 ± 8.2[b,d,f]	36.1 ± 6.1[b,d,f]	23.3 ± 5.2[b,d,f]	31.2 ± 7.8[b,d,f]
Dexamethasone	17.9 ± 5.6[b,d]	19.0 ± 5.2[b,d]	18.7 ± 5.7[b,d]	18.5 ± 5.2[b,d]	15.5 ± 4.3[b,d]	17.6 ± 4.9[b,d]
Acetic acid control	52.5 ± 9.1[b]	74.1 ± 9.5[b]	60.2 ± 8.3[b]	70.7 ± 9.7[b]	44.5 ± 8.9[b]	51.5 ± 9.8[b]
Normal control	7.6 ± 5.7	9.0 ± 4.8	9.1 ± 4.4	9.4 ± 4.9	7.6 ± 4.1	8.1 ± 4.2

[b]$P < 0.01$, *vs* Normal control group; [d]$P < 0.01$, *vs* Acetic acid control group; [f]$P < 0.01$, *vs* Dexamethasone group.

ted with the degree of inflammatory injury (Table 4), and these markers were rarely expressed in samples taken from the normal control group. The positive percentage and density of NF-κB p65, TNF-α and ICAM-1 in injured colon was significantly higher than that in normal control. After DG or dexamethasone treatment, the positive percentage and density of these molecules were reduced significantly, which indicates that both DG and dexamethasone may inhibit the expression of these molecules. Also, the expression of these molecules was significantly lower in DG treated samples than in dexamethasone treated samples ($P < 0.01$).

DISCUSSION

DAI, morphologic injury and histological changes are usually applied to evaluate the severity of inflammatory injuries. MPO reflects the activity of neutrophils, which is also a good indicator of the acute inflammatory reaction[5,6]. According to the indices mentioned above, a rat model of ulcerative colitis (acute phase) was induced by colonic infusion of acetic acid in these experiments. Compared with the acetic acid control group, these inflammatory indices were significantly improved by treatment with DG or dexamethasone.

To understand the molecular mechanism of the effect of DG in this experimental model, the expression of NF-κB, ICAM-1 and TNF-α was detected by immunohistochemistry. All of these molecules are known to be upregulated in the acute inflammatory cascade, which plays an important role in the pathogenesis of ulcerative colitis[7]. Activation of NF-κB is key to the expression of many proinflammatory cytokines and adhesive molecules including ICAM-1 and TNF-α[8-11]. ICAM-1 induces the migration and infiltration of inflammatory cells into the lesion[12,13], while TNF-α causes apoptosis of the colonic mucosa[14,15]. Both ICAM-1 and TNF-α represent different key points in the progress of inflammatory injuries[16,17]. TNF-α, ICAM-1 and NF-κB were upregulated in the acetic acid control group, but reduced significantly after DG or dexamethasone treatment, which means that inhibition of these molecules was likely important for the protective effect of DG and dexamethasone.

As reported previously, NF-κB is a key molecule in both the initiation and progression phase of the inflammatory reaction[18,19]. Activated NF-κB translocates into the nucleus and induces the expression of proinflammatory cytokines, adhesive molecules and chemokines. In this rat model, the expression of NF-κB was inhibited by both DG and dexamethasone, which means that the anti-inflammatory mechanism of DG may be similar to that of dexamethasone. Though the efficacy of DG was less than that of dexamethasone, its side-effects are expected to be less severe.

In summary, DG was efficacious in experimental ulcerative colitis induced in rats, and associated with insignificant side effects. This result suggests that DG may be a promising drug candidate for the treatment of ulcerative colitis.

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RAPID COMMUNICATION

Expression of vascular endothelial growth factor-C and angiogenesis in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of vascular endothelial growth factor-c (VEGF-C) mRNA and microvessel density (MVD) in human esophageal squamous cell carcinoma (ESCC) and its relationship with clinical significance.

METHODS: Specimens obtained from 43 patients undergoing surgical resection for ESCC were used in this study. The expression of VEGF-C mRNA was examined by *in situ* hybridization. Tumor MVD was determined immunohistochemically with anti-CD31 antibody and estimated by image analysis. Ten sections of adjacent normal mucosa were also examined.

RESULTS: VEGF-C mRNA expression was detected in cytoplasm of carcinoma cells. Of the 43 ESCC patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA. No VEGF-C mRNA expression was observed in normal esophageal mucosa. VEGF-C mRNA expression correlated significantly with lymph node metastasis, TNM stage and depth of invasion ($P < 0.05$). Furthermore, histological grade (differentiation) tended to correlate with VEGF-C mRNA expression, but was not statistically significant ($P > 0.05$). In tumor lesions, the MVD was significantly greater than that in normal esophageal mucosa. MVD correlated significantly with lymph node metastasis, TNM stage and depth of invasion ($P < 0.05$), but not with histological grade (differentiation) ($P > 0.05$). Lesions with VEGF-C mRNA expression had a significantly higher MVD than that of those without VEGF-C mRNA expression ($P < 0.05$).

CONCLUSION: VEGF-C plays a role in lymphatic metastasis via lymphangiogenesis and angiogenesis in ESCC. VEGF-C is one of the important predictors of the biological behavior in ESCC.

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Key words: Vascular endothelial growth factor-c; Esophageal carcinoma; Angiogenesis; Microvessel density; Lymph node metastasis; *In situ* hybridization

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INTRODUCTION

Lymph node metastasis is an important prognostic factor for human esophageal squamous cell carcinoma (ESCC)[1]. Angiogenesis induced by a variety of growth factors such as vascular endothelial growth factor (VEGF), has been recently considered necessary for the growth, invasion and metastasis of solid tumors[2]. Thus, measurement of vascular growth may be clinically important in esophageal cancer specimens. To assess angiogenesis, several markers of blood vessel endothelium have been developed for microscopic estimation of microvessel density(MVD) by immunohistochemistry, including CD31/PECAM-1, CD34, and factor Ⅷ-related antigen(von Willebrand factor or vWF)[3]. VEGF-C, a member of the VEGF family, stimulates the proliferation of both vascular and lymphatic endothelial cells via VEGF receptor (VEGFR)-2 and VEGFR-3 in many physiological and pathological processes[2,4]. Previous reports have shown that VEGF-C expression in cancer tissues has a positive correlation with the risk of lymphatic metastasis in a variety of cancers[5] including prostatic[6], gastric[7], oral[8], lung[9], colorectal[10,11] and bladder carcinoma[12]. A similar tendency has been reported for esophageal cancers, although a significant correlation between VEGF-C expression and the frequency of nodal metastasis is not always found[13-15]. Hence, the precise role of VEGF-C in ESCC has not been clearly understood[1]. Therefore, in the present study, VEGF-C mRNA expression in biopsy specimens of ESCC was examined by *in situ* hybridization (ISH) and the association between VEGF-C mRNA expression and clinicopathological factors, including the intratumoral microvessel density (MVD) as a measure of tumor angiogenesis in ESCC was invested.

MATERIALS AND METHODS

Patients and tumor specimens

Tumor specimens from 43 ESCC patients (32 males and 11 females, mean age 62.5 years, range 47-76 years) undergoing surgical treatment in Jinhua People's Hospital from 1997 to 2002 were included in the study. Specimens were classified according to the TNM classification system by UICC and 10 normal esophageal tissues were collected as control. None of the patients received any radiotherapy or chemotherapy prior to the study. Histological grades, depth of invasion and lymph node metastasis are shown in Table 1.

Methods

In situ mRNA hybridization (ISH) analysis was performed as described previously with minor modifications[16]. VEGF-C specific oligonucleotide probes were labeled with digoxin at the 5'-end and synthesized by Beijing Dinguo Co. Ltd (Dingguo, Beijing, China). The DNA oligonucleotide sequences used are 5'-TGTACAAGT GTCAGCTAAGG-3' and 5'-CCACATCTATACACAC CTCC-3'. Tissue sections (4 μm-thick) were incubated with 50 mg/L proteinase K, and then hybridization was performed in a moist chamber for 16 h at 45℃, using a prove concentration of 100 ng/L. The sections were then incubated with a staining solution containing NBT/BCIP in a dark box for 1-2 h. Negative controls ware performed in all cases by omitting the probes.

A mouse CD31 mAb (JC/70A, DAKO Corporation, Denmark) was used as a vascular endothelial marker[3,17]. Immunohistochemical examination for CD31 was performed with EnVision kits to access MVD. In brief, after deparaffinization and rehydration, the sections were denatured for 3 min in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0) for antigen retrieval. Endogenous peroxide activity was quenched with hydrogen peroxide, and the sections were incubated with goat serum for 20 min at room temperature to block nonspecific binding of anti-CD31 mAb (JC/70A, 1:50 dilution) and then for 16 h at 4℃ at 1:50 dilution. Bound antibodies were detected by the EnVision system (DAKO Corporation, Denmark) and using 3, 3'-diamindoenzidine as the chromogen while nuclei were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibodies.

The results of ISH and MVD were examined by two of the authors with no prior knowledge of the identification of each section, using Leica Qwin image system (Leica Corporation, Germany). The expression of VEGF-C mRNA in the tissue was defined as positive if distinct staining was observed in at least 10% of tumor cells[18]. For determination of MVD, the number of microvessels with positive reaction to JC/70A was counted in five randomly selected microcopy fields on the vascular areas within a section (× 200, 0.7386 mm^2/field) and the average was then calculated.

Statistical analysis

The data were evaluated by *t* test, Fisher's exact probability test or chi square test. $P < 0.05$ was considered statistically significant for all tests. All statistical analyses were performed using the SPSS 10.0 statistical package (SPSS, Inc, Chicago, IL).

Table 1 Correlation between VEGF-C mRNA expression in ESCC tissue and clinicopathological factors

Clinicopathological factors	*n*	VEGF-C mRNA *n* (%)	MVD/mm^2 (mean ± SD)
TNM stage			
Ⅰ, Ⅱ	33	11 (33.3)	60.14 ± 20.2
Ⅲ, Ⅳ	10	7 (70.0)[a]	83.68 ± 33.6[a]
Histological grade			
G_1	12	4 (33.3)	60.5 ± 31.7
G_2	20	8 (45.0)	66.30 ± 21.6
G_3	11	6 (54.6)	71.66 ± 30.8
Depth of invasion			
T_1, T_2	23	5 (21.7)	64.24 ± 19.3
T_3, T_4	20	13 (65.0)[b]	68.86 ± 31.7
Lymph node metastasis			
Negative	31	10 (32.3)	58.66 ± 18.8
Positive	12	8 (66.7)[a]	85.30 ± 32.7[a]

[a]$P < 0.05$ *vs* negative lymph node metastasis and TNM stages Ⅲ and Ⅳ; [b]$P < 0.01$ *vs* depth of tumor invasion of T_3 and T_4.

RESULTS

Expression of VEGF-C mRNA in ESCC tissues

Of the 43 patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA expression in cytoplasm. The specimens were divided into two categories by the staining pattern of VEGF-C mRNA, diffuse or focal staining of carcinoma cells (Figures 1A and 1B). In contrast, no VEGF-C mRNA in specimens of adjacent normal esophageal mucosa was stained.

CD 31 expression and MVD in ESCC tissues

Immunohistochemical expression of CD31 was detected in cytoplasm of vascular endothelial cells. Brown capillaries or small clusters standing out sharply from other tissues were found to be microvessels (Figures 2A and 2B). At the deepest invasive site of tumors, the MVD ranging 43-144/mm^2 (76.4 ± 20.3/mm^2) was significantly higher than that in normal esophageal mucosa ($P < 0.01$).

Correlation between VEGF-C mRNA expression and MVD and clinicopatholgical factors

VEGF-C mRNA expression in ESCC tissues was associated with the depth of tumor invasion, TNM stage and lymph node metastasis ($P < 0.05$), but not with histological grade ($P > 0.05$). Furthermore, MVD also correlated with TNM stage and lymph node metastasis in ESCC tissue ($P < 0.05$). No association was found between MVD and depth of tumor invasion and histological grade ($P > 0.05$). The patients were divided into VEGF-C mRNA positive and negative groups. The VEGF-C mRNA positive cases showed a higher MVD (79.5 ± 30.9/mm^2) than the VEGF-C mRNA negative cases (57.5 ± 18.4/mm^2) ($P < 0.05$) (Table 1).

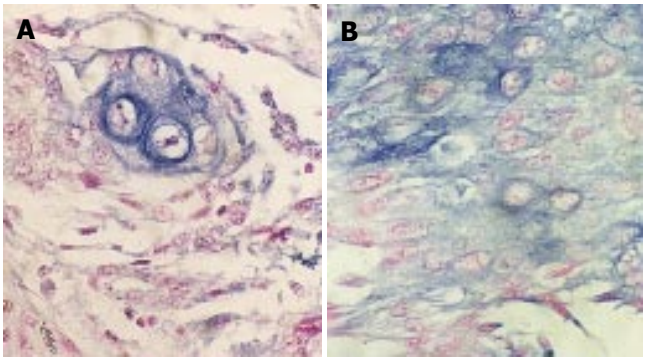

Figure 1 Focal (**A**) and diffuse (**B**) expression of VEGF-C mRNA in ESCC tissue as blue granules in cytoplasm of tumor cells (*in situ* hybridization × 400).

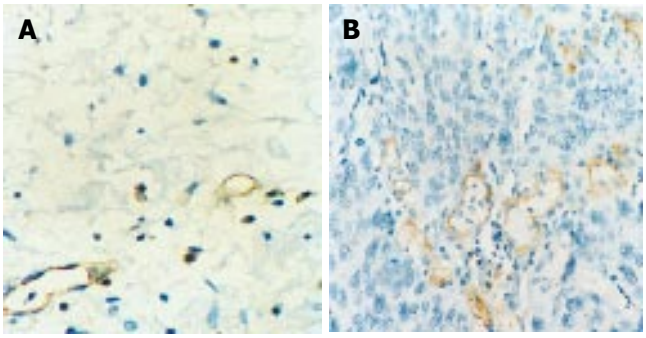

Figure 2 Immunohistochemical staining of CD 31 in normal esophageal (**A**) and ESCC (**B**) tissue (EnVision × 400).

DISCUSSION

Recent studies have shown a significant relation between MVD, VEGF expression and prognosis in a variety of tumors including esophageal carcinoma[19]. Among the VEGF family members, VEGF-C appears to affect the lymphatic system as a lignant for VEGFK-3, which is specifically expressed on lymphatic vessels, and VEGF-C is suspected to play an important role in lymphangiogenesis[2,4,5]. But little is known about the mechanisms of lymphangiogenesis and lymphatic metastasis, while the effects of VEGF-C in ESCC remain controversial[1]. In this study, we investigated the clinicopathalogical significance of VEGF-C mRNA expression in relation to the angiogenesis in ESCC. The results showed that 41.9% of patients with ESCC had expression of VEGF-C mRNA, which correlated significantly with lymph mode metastasis, depth of tumor invasion and TNM stage, suggesting that VEGF-C plays a role in ESCC. It was reported that VEGF-C can be detected using immunohistochemical staining and RT-PCR, and VEGF-C expression in ESCC may play a key role in tumor progression and lymphatic metastasis[13,14]. It was also reported that only histological grade, but not parameters involved in lymphatic spread, correlates with VEGF-C expression in ESCC[15]. These contradictory findings can be explained by difference in analytic method. Even in immunohistochemial analysis, the result may differ depending on the selected site for assessment.

More interestingly, we found that VEGF-C mRNA expression was significantly correlated with MVD, indicating the grade of angiogenesis in ESCC. Tsurusaki *et al*[6] and Akagi *et al*[18] have failed to find any remarkable differences in blood vessel density between VEGF-C positive and negative prostatic and colorectal carcinoma specimens. Also, VEGF-C expression is not correlated with MVD in human gallbladder cancer[20]. Whereas other studies reported that VEGF-C is associated with angiogenesis in breast cancer[21], colorectal carcinoma[22,23] and bladder transitional cell carcinoma[12], and plays an important role in angiogenesis and lymphangiogenesis in lung cancer[9] and malignant mesothelioma[24]. It has been reported that VEGF-C can bind to both VEGFR-2 and VEGFK-3[25]. The specificity of VEGF-C for its 2 receptor is known to be regulated by proteolytic processing. Accordingly, only fully processed VEGF-C activates both VEGFR-2 and VEGFK-3, whereas the partially processed precursors act only through VEGFK-3[26]. Activation of VEGFR-2 results in mitogenesis of vascular endothelial cells, whereas VEGFR-3 activation by VEGF-C is considered to induce proliferation of lymphatic endothelial cells. Furthermore, recent studies have revealed that VEGFR-3 is expressed in angiogenic blood vessels in certain pathological conditions. For example, VEGFR-3 expression can detected in intratumor blood vessels of human breast cancer[21], cutaneous melanoma[27], head and neck squamous cell carcinoma[28], gliomas as well as in vascular endothelial cells activated during formation of granulation tissue in skin[29]. Thus, angiogenesis and lymphangiogenesis responses to VEGF-C have been found to depend on the expression of its receptors in blood and lymphatic endothelial cells of the target tissue.

In our present study, patients with VEGF-C mRNA expression had a significantly higher MVD that those without VEGF-C mRNA expression, suggesting that VEGF-C secreted from tumor cells may stimulate not only lymphangiogenesis but also angiogenesis, which are significantly correlated with lymph node metastasis in ESCC. As a rule, once cancer cells enter blood microvessels, they can reach regional lymph nodes through lymphatic vessels from blood vessels, via blood vessel-lymph vessel junctions. In fact, the lymphatic and vascular systems have numerous connections allowing disseminating tumor cells to pass rapidly from one system to the other. Furthermore, metastatic tumor cells expressing VEGF-C could grow in regional lymph nodes that they reached.

In conclusion, VEGF-C mRNA is expressed heterogeneously in tumor cells and plays an important role in angiogenesis and lymphangiogenesis, as well as growth and metastasis of ESCC. The combined examination of VEGF-C expression and MVD in biopsy specimens from ESCC patients can predict lymph node metastasis and select appropriate treatments.

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CASE REPORT

Diagnosis of autoimmune gastritis by high resolution magnification endoscopy

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Abstract

Endoscopic visualisation of gastric atrophy is usually not feasible with conventional endoscopy. Magnifying endoscopy is helpful to analyze the subepithelial microvascular architecture as well as the mucosal surface microstructure without tissue biopsy. Using this technique we were able to describe the normal gastric microvasculature pattern and we also identified characteristic patterns in two cases of autoimmune atrophic gastritis.

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Key words: Magnification endoscopy; Autoimmune gastritis; Collecting venules; Subepithelial capillary network

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INTRODUCTION

Endoscopic visualisation of gastric atrophy is usually not feasible with conventional endoscopy. Except for the absence of rugae and visible vessels in the gastric corpus, macroscopic features, as observed during gastroscopy, are of very limited value in the evaluation of the presence of gastric atrophy[1]. Although histology may be considered as a gold standard for detection of gastric atrophy, neither the original nor the revised version of the Sydney system reliably identifies more than half of the cases in patients with confirmed atrophy[2].

Magnifying endoscopy is a helpful procedure to analyze the subepithelial microvascular architecture as well as the mucosal surface microstructure without tissue biopsy[3]. Using this technique we were able to observe the normal gastric microvasculature pattern and we also identified characteristic patterns in two cases of autoimmune atrophic gastritis.

CASE REPORT

Two male patients (aged 53 and 62 years), underwent upper gastrointestinal endoscopy due to anaemia and low serum B12 levels. Endoscopy was performed using the GIF-Q240Z (Olympus, Keymed, UK) high-resolution magnifying endoscope (× 115 on a 20" screen, 7.9 μm resolution). Prior to endoscopy, a soft black hood was mounted on the tip of the scope, to enable the endoscopist to fix the focal distance at 2 mm between the tip of the scope and the gastric mucosa. Ordinary endoscopic findings were normal, however, magnified observation of the gastric mucosa showed normal gastric antral microvasculature (coil-shaped subepithelial capillary network (SECN) pattern) (Figure 1A), with disappearance of the normal honeycomb-like SECN and irregular collecting venules and areas of tubular structures in the gastric body mucosa (Figures 1B). Targeted biopsies from the gastric body showed glandular atrophy, intestinal metaplasia and inflammatory infiltrate in the gastric corpus, sparing the antrum, as in autoimmune gastritis (Figure 1C). On the other hand, the histological findings in the biopsied specimens from the antral mucosa showed no pathological changes. Histology showed no Helicobacter pylori micro-organisms. Serum anti-parietal cell antibodies were positive.

DISCUSSION

Autoimmune gastritis is characterized by autoimmune destruction of fundic and body glands. The marker for the most severe, end-stage form of diffuse corporal atrophic gastritis is pernicious anemia[4]. However, no endoscopic findings specific for this entity have been reported.

The microvascular pattern of human gastric mucosa has recently been investigated[5-7]. The normal gastric body and fundus mucosal microvessels show two major components: (1) subepithelial capillaries; and (2) collecting venules. Polygonal loops of subepithelial capillaries surround the neck of gastric pits. These loops form a honeycomb-like SECN and converge onto mucosal collecting

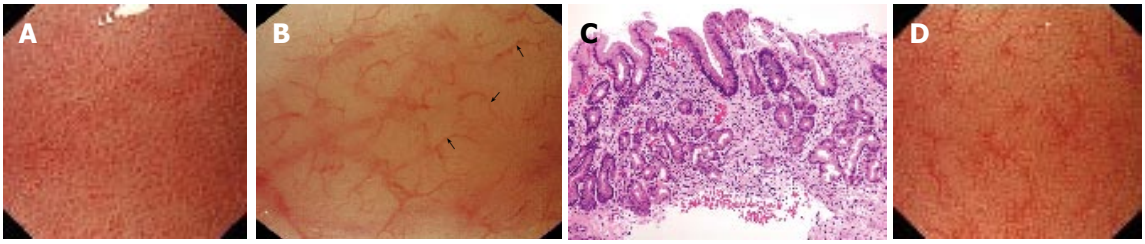

Figure 1 **A**: Magnified view of gastric antral mucosa in our patient. All parts of the antral mucosa demonstrated a coil-shaped subepithelial capillary network (SECN) in regular arrangement; **B**: Magnified endoscopic view of the gastric body mucosa in our patient. Loss of the normal SECN and collecting venules (arrows) in irregular shape and arrangement were evident. These findings are compatible with those of gastric atrophy; **C**: Histology reveales decreased density of glands, loss of specialised glands in the gastric body; **D**: Magnified view of normal gastric body mucosa in another healthy patient Honeycomb-like SECN with collecting venules in regular arrangement can be seen clearly.

venules that drain down into the submucosa (Figure 1D). In the antral mucosa, the normal subepithelial capillaries form a coil-shaped network, while collecting venules are rarely seen.

Yao *et al* have demonstrated that the resolution of the GIF-Q240Z gastroscope is 7.9 μm. Since the minimal diameter of subepithelial capillaries in the gastric mucosa, as described in anatomic studies, is 8 μm, this endoscope is ideally suited to visualize microvessels and capillaries[6].

Nakagawa *et al* have recently studied the usefulness of magnifying endoscopy for the diagnosis of *H pylori*-induced histopathologic gastritis[8]. The observed morphology of collecting venules was divided into the 3 patterns: (1) regular (R), which had the qualities of regularity in venules size, visible second or third order branches, and a uniform distance between venules; (2) irregular (I), which had the qualities of irregularity in size, inability to observe second or third order branches, and a lack of a uniform distance between the collecting venules, with venules sometimes fused to adjacent venules and sometimes lying horizontally; and (3) obscured (O), in which no collecting venules were visible. Observation of an R pattern indicates an absence of *H pylori* infection and histopathologic gastritis. Observation of an O or I pattern indicates the presence of histopathologic gastritis from *H pylori* infection, and an I pattern suggests the presence of gastric mucosal atrophy. We have also shown that in cases of gastric atrophy associated with *H pylori* infection, the normal honeycomb-like subepithelial capillary network (SECN) in gastric body disappears and the collecting venules become irregular[9].

The magnified endoscopic findings of diffuse atrophy in gastric body mucosa and normal antrum associated with autoimmune gastritis have not yet been described. In our cases, the magnified views in gastric antrum revealed a normal coil-shaped subepithelial capillary network. On the other hand, in gastric body mucosa we demonstrated loss of the normal honeycomb-like SECN pattern with irregular collecting venules, suggestive of corporal gastric atrophy, as well as areas with tubulovillous structures as seen in intestinal metaplasia. These magnified endoscopic findings were constantly detected in all parts of the gastric corpus that were examined. These patterns are suggested to be characteristic of autoimmune gastritis and were confirmed by histology.

In summary, it seems that irregular collecting venules can be recognised both in autoimmune- and *H pylori*-associated gastric atrophy. The dissemination of this finding can be used to help differentiate between these two types of gastritis. Gastric body predominant atrophy visualised by magnifying endoscopy, can be very useful in making a precise diagnosis of autoimmune gastritis.

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CASE REPORT

Coexistence of esophageal superficial carcinoma and multiple leiomyomas: A case report

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Abstract

Leiomyomas are the most common benign tumors of the esophagus. They usually occur as a single lesion or as two or three nodules. Only two cases of esophageal multiple leiomyomas comprising more than 10 nodules have been reported previously. Moreover, there have been few reports of esophageal squamous cell carcinoma overlying submucosal tumors. We describe a 71-year-old man who was diagnosed as having a superficial esophageal cancer coexisting with two or three leiomyoma nodules. During surgery, 10 or more nodules that had not been evident preoperatively were palpable in the submucosal and muscular layers throughout the esophagus. As intramural metastasis of the esophageal cancer was suspected, we considered additional lymphadenectomy, but had to rule out this option because of the patient's severe anoxemia. Microscopic examination revealed that all the nodules were leiomyomas (20 lesions, up to 3 cm in diameter), and that invasion of the carcinoma cells was limited to the submucosal layer overlying a relatively large leiomyoma. This is the first report of superficial esophageal cancer coexisting with numerous solitary leiomyomas. Multiple minute leiomyomas are often misdiagnosed as intramural metastasis, and a leiomyoma at the base of a carcinoma lesion can also be misdiagnosed as tumor invasion. The present case shows that accurate diagnosis is required for the management of patients with coexisting superficial esophageal cancer and multiple leiomyomas.

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Key words: Multiple leiomyomas; Squamous cell carcinoma; Esophagus; Coexistence

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INTRODUCTION

Leiomyomas are the most common benign tumors of the esophagus, accounting for roughly two-thirds of all benign tumors of this organ. Although its overall incidence reported in autopsy ranges from 0.005% to 5%,[1,2] this may be an underestimation because many small lesions are missed at autopsy unless the esophagus is examined carefully. Studies involving detailed histological examinations have reported the frequency to be higher, at 7.9%[3]. Since esophageal leiomyomas are generally slow-growing and the size of tumors may not change for many years, most affected patients are asymptomatic. Considering the risk of surgery and the patient's quality of life, non-surgical management is recommended.

Surgical resection of esophageal leiomyomas is warranted only when: (1) there is severe dysphagia due to giant or multiple lesions, (2) there is a possibility of symptomatic transformation, (3) there is a need to obtain a definitive histological diagnosis, (4) malignancy can only be ruled out by surgical removal, or (5) malignant epithelial tumors are also present.

Both giant and multiple lesions involving the entire esophagus can cause severe and debilitating dysphagia. The presence of a giant lesion itself is a risk factor for malignancy, and such lesions should be treated surgically even if they cause no symptoms. Otherwise, esophageal leiomyoma usually appears as a solitary tumor (97%). Occasionally (less than 2.4% of all cases), more than one esophageal leiomyoma; i.e., generally two or three, may occur in the same patient. Multiple leiomyomas of the esophagus are extremely rare, and only two such cases, involving more than 10 nodules, have been reported

previously[4,5]. Diagnosis of medium-sized leiomyomas is generally easy using endoscopic ultrasonography (EUS) and computerized tomography (CT). However, this is difficult when multiple leiomyomas coexist with carcinoma lesions. In cases where carcinoma overlies a submucosal leiomyoma, there is a possibility of overestimating the extent of tumor invasion, and multiple minute leiomyomas are sometimes misdiagnosed as intramural metastasis.

Here, we describe a case of superficial esophageal squamous cell carcinoma (SCC) coexisting with multiple (more than 20) leiomyoma lesions, and we discuss the management of such patients and the histogenesis of carcinomas overlying esophageal submucosal tumors (SMT).

CASE REPORT

The patient is a 71-year-old man who presented with a cervical tumor in October 2000. He was diagnosed as having a malignant lymphoma of the thyroid and esophageal leiomyomas. The thyroid tumor disappeared completely after chemotherapy, and the patient maintained a status of complete remission. Barium swallow esophagograms showed two smoothly rounded defects. The larger tumor, 40 mm in diameter and exhibiting two or three bumps, was located in the middle third of the esophagus. The smaller one, 20 mm in diameter, was in the lower third of the esophagus (Figure 1A). Both tumors were covered by normal mucosa, and showed no signs of malignancy in CT and EUS examinations. Therefore, we diagnosed these SMTs as two (or possibly three) leiomyomas. One year later, in December 2001, no marked changes were evident in a barium swallow examination (Figure 1B). However, endoscopy revealed a superficial ulcerative tumor (24 mm in diameter) in the lower third of the esophagus, overlying the smaller SMT (Figure 2: Left). Chromoendoscopy with Lugol's iodine solution demonstrated that the polypoid lesion was located within the non-staining area and that part of the surface was covered by normal mucosa (Figure 2: Right). Multiple biopsies revealed that the ulcerative tumor was SCC. EUS demonstrated that the cancer overlaid one of the leiomyomas, originating in the muscular layer, suggesting that the cancer may have invaded only as far as the submucosal layer. In February 2002, the patient underwent esophagectomy with a gastric pull-up reconstruction and cervical anastomosis via abdominal, left cervical and right thoracotomy incisions at the Department of Surgery I, Iwate Medical University School of Medicine, Morioka, Japan. During the operation, 10 or more nodules that had not been evident preoperatively were palpable in the submucosal and muscular layers throughout the esophagus. Intramural metastasis of the esophageal cancer was therefore suspected, and additional lymphadenectomy was considered. However, this option was ruled out because of the patient's severe anoxemia.

The resected specimen showed a superficial ulcerative tumor and multiple SMTs of various sizes (Figure 3). Macroscopically, about 10 SMTs were evident throughout the esophagus. Each SMT appeared as a well demarcated round nodule, and none of the tumors showed continuity with others, which is a characteristic feature of leiomyomatosis. Microscopically, more than 20 SMTs, measuring from 1 mm to 3 cm in diameter, were scattered throughout the esophagus (Figures 4 and 5: Arrows). All the SMTs showed low overall cellularity, and were composed of interlaced smooth-muscle cells with hypovascularity and no mitosis (Figure 5B). These findings are characteristic of leiomyoma. The ulcerative tumor overlaid one of the leiomyoma nodules in the lower third of the esophagus. The tumor was confirmed as SCC, and

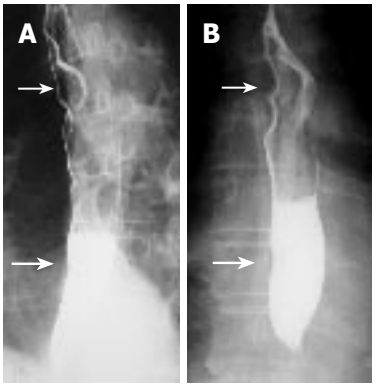


Figure 1 Barium swallow esophagograms. Lack of any marked change in two smooth round defects over the course of a year. **A**: On admission; **B**: One year later.

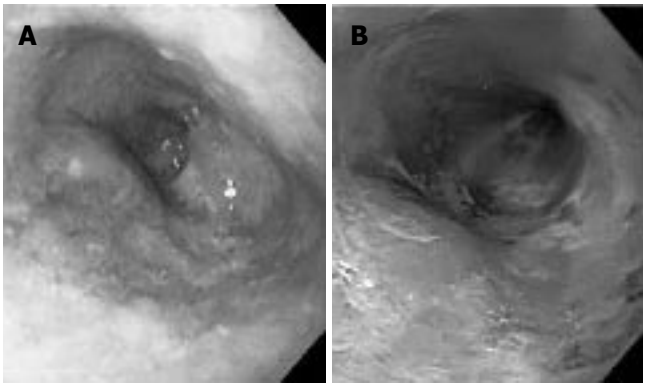


Figure 2 **A**: Endoscopic examination demonstrates a polypoid lesion with a partially irregular surface; **B**: Chromoendoscopy with Lugol's iodine solution demonstrates a polypoid lesion located within the non-staining area and normal mucosa covering part of the surface.

Figure 3 Resected specimen. Multiple polypoid tumors of various sizes are covered with normal mucosa (circles). A polypoid lesion in the lower third of the esophagus exhibits an irregular surface. The broken line indicates the area of SCC.

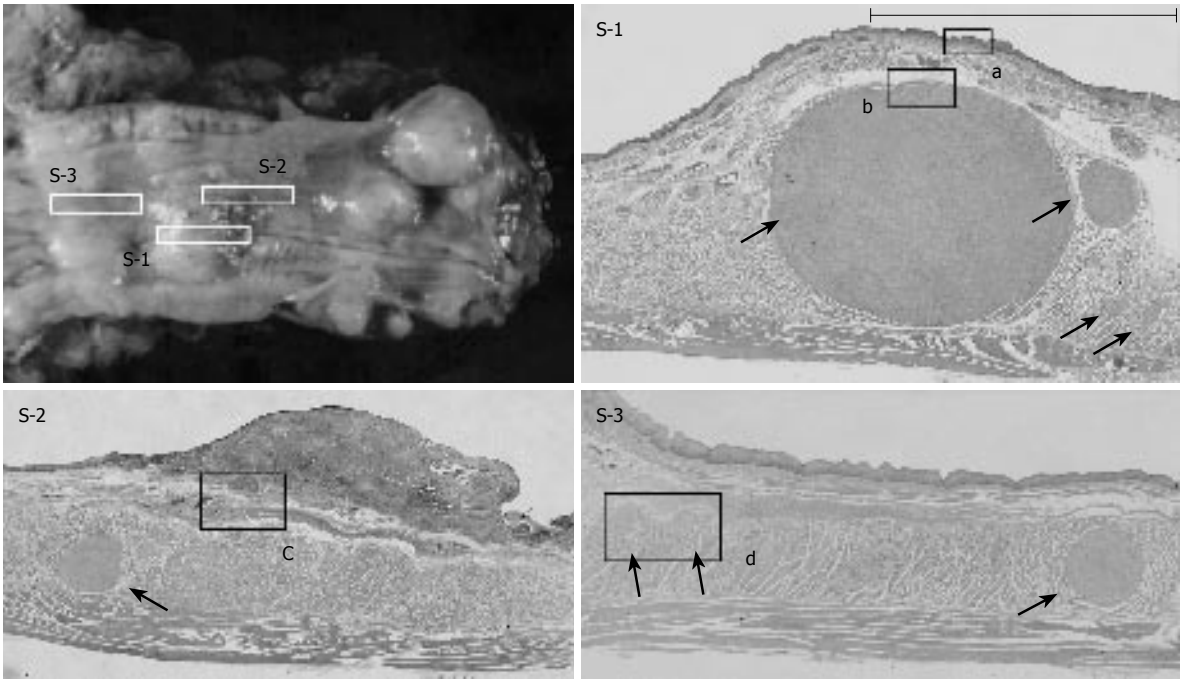
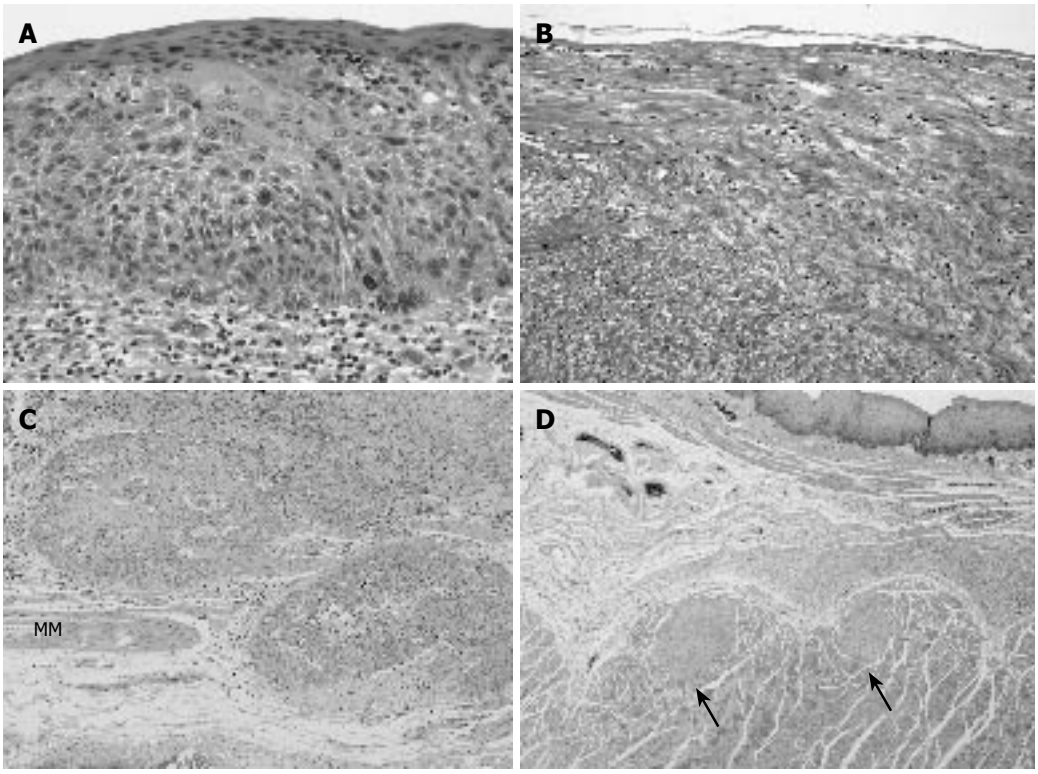


Figure 4 Photomicrograph of cross-sections (squares) of the resected specimen. Leiomyoma tissue is located in all sections (arrows). **S-1**: CIS overlies the leiomyoma. The region of CIS extends to the oral side of the leiomyoma (bar). **S-2**: SCC invades the submucosal layer in a very narrow region. **S-3**: A section without abnormal macroscopic findings. Multiple small leiomyomas are observed only by microscopic examination.



extensive carcinoma in situ (CIS) was observed on the leiomyoma (Figures 4 S-1, 5A). On the oral side of the SMT, the cancer had invaded into the submucosal layer, without lymph node metastasis (Figures 4 S-2, 5C).

The patient was discharged one month after the operation. At the time of writing, three years later, no local recurrence or distant metastasis of the SCC or malignant lymphoma has been observed.

DISCUSSION

As a distinct entity, multiple leiomyoma of the esophagus involving solitary lesions needs to be clearly distinguished from diffuse leiomyomatosis. Multiple leiomyoma of the esophagus is extremely rare, and only two such cases, involving more than 10 lesions, have been reported previously[4,5]. Bradford *et al* reported a patient with 14

Table 1 Cases of ESC located in the surface epithelium over benign tumors

	Authors	Age	Sex	Submucosal tumor (SMT)	Size of SMT (cm)	Depth of cancer invasion	Subviews and <Management>
1	Marcial-Rojas RA	63	Male	Lipoma	11 × 3.5	Mucosal layer	<Cervical esophagotomy, excision>
2	Iizuka *et al*	75	Male	Leiomyoma	3.0 × 2.0	Mucosal layer	<Blunt dissection>
3	Watanabe *et al*	53	Male	Leiomyoma	0.8 × 0.6 × 0.25	Intraepithelium	Dysplasia <Chemotherapy,esophagectomy>
4	Callanan *et al*	54	Male	Leiomyoma	1.25	Muscular layer	<Esophagectomy>
5		45	Male	Leiomyoma	4	Muscular layer	<Esophagectomy>
6	Kuwano *et al*	63	Male	Leiomyoma	1	Mucosal layer	Dysplasia <Esophagectomy>
7		71	Male	Lipoma	1.5	Mucosal layer	<Esophagectomy>
8	Yosikane *et al*	52	Male	Leiomyoma	1.5 × 1.1 × 0.7	Mucosal layer	CIS and dysplasia <Esophagectomy>
9		67	Male	Lipoma	2.8 × 4.5	Intraepithelium	Separate cancer <Esophagectomy>
10	Nagashima *et al*	61	Male	Leiomyoma	2.9 × 2.0	Lamina propria	<Endoscopic resection>
11	Mizobuchi *et al*	64	Male	Leiomyoma	0.7 × 0.65	Lamina propria	<Esophagectomy>
12	Fu *et al*	62	Male	Leiomyoma	2	Mucosal layer	<Endoscopic resection>
13	Present case	73	Male	Leiomyoma	2.0 × 1.5	Submucosal layer	Multiple leiomyomas <Esophagectomy>

esophageal leiomyomas measuring 1 to 4 cm in diameter, and Seremetis *et al* reported another patient who had 15 such leiomyomas 0.4 to 6 cm in diameter. In our patient, more than 20 leiomyomas, measuring from 1 mm to 3 cm in diameter, were scattered throughout the esophagus.

There have been several reports of esophageal SCC coexisting with benign SMT[6-14]. This presentation shows two types: one is the overlying type, where the carcinoma covers the benign SMT, and in the other type the two lesions are separate. Generally, most SMTs in the separate type are minute leiomyomas that are discovered only during postoperative examination of pathology specimens[3]. This type of presentation is often encountered during esophagectomy for esophageal SCC. Otherwise, twelve cases of the overlying type have been reported previously[6-14] and are summarized in Table 1. Although benign tumors of various sizes have been reported, all of them protruded into the esophageal lumen. Some investigators have speculated that the pathogenesis of the overlying type may involve chronic irritation of the esophageal mucosa, due to intraluminal protrusion of the SMT, leading to carcinoma development[9-11]. This hypothesis has been suggested in reports of SCC coexisting with lipomas as well as leiomyomas[15]. In the present patient, both overlying and separate types were observed. Although there were 20 leiomyomas scattered throughout the esophagus, the SCC overlaid a relatively large and protruding tumor. Thus, the size and location of SMTs might affect the degree to which the esophageal mucosa is exposed to chronic irritation.

Recently, curative endoscopic resection has been performed for patients with carcinomas overlying SMTs. However, such cases require accurate diagnosis of the depth of invasion using EUS[7,8]. Mizobuchi *et al* reported a case in which an early esophageal SCC was overdiagnosed because the leiomyoma appeared to be a component of the carcinoma[6]. In these reports, the authors emphasized that accurate diagnosis of the leiomyoma made it possible to avoid excessive surgery. Several diagnostic modalities, such as ballium swallow, endoscopy, CT and EUS, allow esophageal leiomyoma to be diagnosed effectively, and most of them have been monitored[16]. With these examinations, however, small leiomyomas are often difficult to diagnose. Indeed, as in the present case, a number of small SMTs may not be apparent preoperatively. During surgery, we were unable to completely rule out the possibility that some of the SMTs were intramural metastases of the ESC, as multiple leiomyoma of the esophagus is quite rare and intramural metastasis of esophageal superficial carcinoma is relatively common.

Since many coexisting carcinomas reported previously have been superficial, it has been speculated that the coexisting SMT may in fact inhibit the growth and invasion of the carcinoma[10]. In the present case, the overlying SCC had invaded only as far as the mucosal layer, and submucosal invasion was limited to only a very narrow region where the SMT was absent. This case appears to support the speculation that SMT inhibits the growth of SCC. During the operation, lymphadenectomy was ruled out because of the patient's anoxemia. If conditions had been more favorable, the patient might have undergone more aggressive surgery. Therefore, in cases where a coexisting superficial carcinoma overlies a benign SMT, a more noninvasive therapeutic procedure (EMR, thoracoscopic approach, and omission of lymphadenectomy *etc.*) should be chosen.

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CASE REPORT

A case of gallbladder carcinoma associated with pancreatobiliary reflux in the absence of a pancreaticobiliary maljunction: A hint for early diagnosis of gallbladder carcinoma

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Abstract

A 62-year-old man with progressive thickening of the gallbladder wall visited our outpatient clinic. The biliary amylase level in the common bile duct was 19900 IU/L and that of the gallbladder was 127000 IU/L, although endoscopic retrograde cholangiopancreatography revealed no pancreaticobiliary maljunction. Histology demonstrated a moderately differentiated adenocarcinoma of the gallbladder. Pancreatobiliary reflux and associated gallbladder carcinoma were confirmed in the present case, in the absence of a pancreaticobiliary maljunction. Earlier detection of the pancreatobiliary reflux and progressive thickening of the gallbladder wall might have led to an earlier resection of the gallbladder and improved this patient's poor prognosis.

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Key words: Amylase; Bile; Gallbladder carcinoma; Pancreatobiliary reflux; Pancreaticobiliary maljunction; Diagnosis

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INTRODUCTION

Regurgitation of pancreatic juice into the biliary tract (pancreatobiliary reflux) usually occurs in patients with pancreaticobiliary maljunction and is closely related to the occurrence of biliary malignancy[1,2]. Bile mixed with pancreatic juice is known to induce cellular proliferation and stimulate genetic alterations of the biliary tract epithelium, leading to hyperplasia, dysplasia and ultimately, carcinoma of the biliary tract mucosa[3].

Here we describe a patient with pancreatobiliary reflux in the absence of pancreaticobiliary maljunction, who showed progressive thickening of the gallbladder wall, which developed into an advanced gallbladder carcinoma.

CASE REPORT

A 62-year-old man visited our outpatient clinic for further evaluation of diffuse thickening of the gallbladder wall, detected by ultrasonography, at an annual medical check-up. He had no symptoms, and the physical examination showed no abnormalities. Laboratory test results were within normal limits. The serum levels of carcinoembryonic antigen was 1.3 ng/mL and the carbohydrate antigen 19-9 level was 1.0 U/mL; that is, both were within normal limits. Computed tomography (CT) revealed diffuse thickening of the gallbladder wall and dilatation of the common bile duct to 12 mm (Figure 1A). The patient was followed, and CT was scheduled for 3 mo later. He returned 42 m later with persistent right hypochondralgia and slight tenderness in the right upper quadrant. Laboratory tests, including serum amylase and lipase, were within the normal range. The serum levels of carcinoembryonic antigen was up to 2.1 ng/mL and the carbohydrate antigen 19-9 level increased to 5.0 U/mL, but both were still within the normal limits. CT revealed progressive thickening of the gallbladder wall and carcinoma of the gallbladder was suspected (Figure 1B). Endoscopic retrograde cholangiopancreatography (ERCP) revealed no pancreaticobiliary maljunction, because the length of the common channel was 5 mm, sphincter of Oddi affected the pancreaticobiliary junction and a connection between two the ducts was not visible during the contraction phase of sphincter of Oddi (Figure 2A, B). The biliary amylase level in the common bile duct, sampled during ERCP, was 19900 IU/L. Extended cholecystectomy, bile duct resection, and lymph node dissection were performed. The amylase level in gallbladder bile, sampled during cholecystectomy, was 127000 IU/L. Amylase was measured by an enzymatic method using the substrate 3-ketobutylidenβ-2-chloro-4-nitrophenyl-maltopentaoside (Diacolor Neonate; Toyobo, Osaka, Japan), and the normal range of serum amylase at our institution was 130-400 IU/L. The histopathologic di-

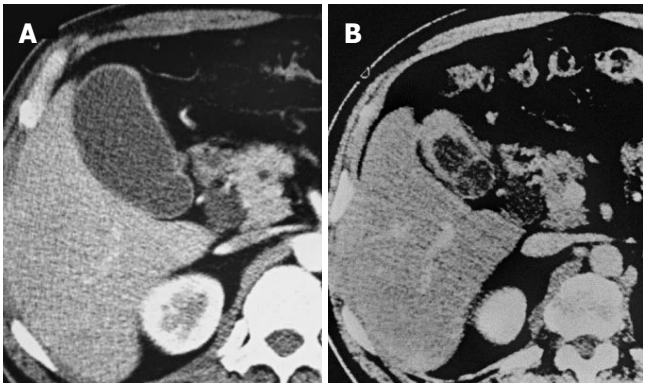

Figure 1 **A:** CT reveals diffuse thickening of the gallbladder wall and dilatation of the common bile duct to 14 mm; **B**: After 42 mo, CT reveals progressive thickening of the gallbladder wall, suspicious for gallbladder carcinoma.

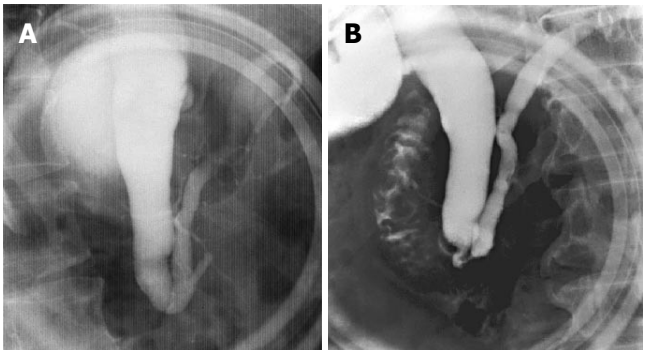

Figure 2 **A** ERCP shows no pancreaticobiliary maljunction, because sphincter of Oddi affected pancreaticobiliary junction and connection between pancreatic and biliary ducts was not visible during the contraction phase of sphincter of Oddi; **B**: ERCP in the relaxation phase of sphincter of Oddi shows the length of the common channel to be 5 mm long.

agnosis of the resected specimen was a moderately differentiated adenocarcinoma of the gallbladder's fundus with invasion of the serosa (Figure 3).

DISCUSSION

Pancreaticobiliary maljunction is defined as an abnormal union of the pancreatic and biliary ducts that is located outside the duodenal wall, where a sphincter system is not present[1]. Thus, two ducts are always communicating, and pancreatic juice freely regurgitates into the biliary tract through this passage. Numerous clinical and experimental studies have supported a relationship between pancreatobiliary reflux and biliary cancer in patients with pancreaticobiliary maljunction. Bile mixed with pancreatic juice is known to induce hyperplasia, metaplasia, and ultimately, carcinoma of the biliary tract mucosa in patients with pancreaticobiliary maljunction[2,3]. In fact, biliary cancer is found in 15% to 67% of adult patients with pancreaticobiliary maljunction, and gallbladder carcinoma is found more often in patients with pancreaticobiliary maljunction without a congenital choledochal cyst[4,5].

Pancreatobiliary reflux also occurred in our patient, in the absence of a pancreaticobiliary maljunction that was confirmed by elevated amylase levels in the bile sampled at

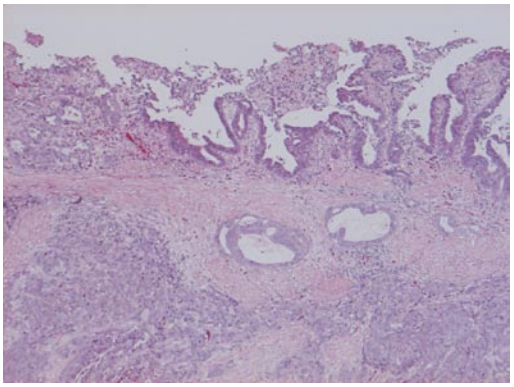

Figure 3 Histopathologic examination of the resected specimen demonstrates a moderately differentiated adenocarcinoma of the gallbladder (HE stain, × 40).

ERCP and cholecystectomy. It was suspected that the extremely high levels of pancreatic enzymes in the bile might have induced carcinoma of the gallbladder as seen in patients with pancreaticobiliary maljunction[4,5]. It was speculated that pancreatic juice may regurgitate into the biliary system through a common terminal ampulla that is known to exist in 60%-90% of human subjects[6]; as pressure generated in the pancreatic duct is usually greater than that observed in the biliary system, it allows for flow from the pancreas to the biliary system[7,8]. The precise mechanism of the pancreatobiliary reflux without pancreaticobiliary maljunction, and especially its relation to the dysfunction of sphincter of Oddi, should be further clarified.

One of the imaging findings that suggest mucosal changes accompanied by pancreatobiliary reflux is diffuse thickening of the gallbladder wall that might reflect cellular proliferation of the gallbladder epithelium, in patients with pancreaticobiliary maljunction[2]. In the present case, diffuse thickening of the gallbladder wall was confirmed on ultrasonography and CT, and its relation to pancreatobiliary reflux could be suggested.

In the present case, pancreatobiliary reflux and associated gallbladder carcinoma were confirmed, in the absence of pancreaticobiliary maljunction. Earlier detection of the pancreatobiliary reflux and progressive thickening of the gallbladder wall might have led to earlier resection of the gallbladder and improved this patient's poor prognosis.

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CASE REPORT

A case of peribiliary cysts accompanying bile duct carcinoma

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Abstract

A rare case of peribiliary cysts accompanying bile duct carcinoma is presented. A 54-year-old man was diagnosed as having lower bile duct carcinoma and peribiliary cysts by diagnostic imaging. He underwent pylorus preserving pancreatoduodenectomy. As for the peribiliary cysts, a course of observation was taken. Over surgery due to misdiagnosis of patients with biliary malignancy accompanied by peribiliary cysts should be avoided.

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Key words: Peribiliary cysts; Bile duct carcinoma; Intrahepatic cholangiocarcinoma

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INTRODUCTION

Hepatic peribiliary cysts are a poorly recognized liver disease characterized by multiple tiny cysts along the portal radicle. This type of lesion is derived from the cystic dilatation of intrahepatic extramural peribiliary glands around the intrahepatic large bile ducts[1]. Although peribiliary cysts sometimes cause stricture of the intrahepatic bile duct and mimics intrahepatic cholangiocarcinoma[2], to our knowledge there are no reports of this condition accompanying bile duct carcinoma. We report a rare case of peribiliary cysts accompanying bile duct carcinoma.

CASE REPORT

A 54-year-old Japanese man was admitted to a local hospital for complaints of jaundice. He was diagnosed with lower bile duct carcinoma and referred to our hospital after placement of endoscopic naso-biliary drainage. He had a history of heavy alcohol use and had been hospitalized for one year with alcoholism ten years previously. Physical examination results were generally unremarkable. The liver was palpable two fingerbreadths below the right costal margin. No ascites or edema was present. Laboratory examination revealed a normal complete blood count and normal coagulation. Liver function test results at admission to our hospital were as follows: aspartate aminotransferase (AST), 75 U/I; alanine aminotransferase (ALT), 133 U/I; alkaline phosphatase (ALP), 467 U; γ-glutamyl transpeptidase (GTP), 196 U/I; total bilirubin, 1.1 mg/mL; indocyanine green test (at 15 min), 6.2%. Hepatits virus screening was negative and no tumor markers were elevated. Abdominal ultrasonography revealed multiple small cystic lesions and tubular anechoic areas along the umbilical portion of the portal vein that were difficult to differentiate with dilatation of the intrahepatic bile ducts. CT revealed a tumor in the lower bile duct and multiple small cystic lesions along the umbilical portion of the portal vein (Figure 1). Cholangiography *via* an endoscopic naso-biliary drainage tube revealed complete obstruction of the lower bile duct, smooth stenosis of the left hepatic duct, and a slight dilatation of the intrahepatic bile duct in the left lobe (Figure 2). Magnetic resonance cholangiopancreatography (MRCP) showed bead-like cystic lesions along the biliary tree in the left lobe of the liver (Figure 3). The left hepatic duct stenosis appeared to be caused by extramural compression by these cysts. Portal phase superior mesenteric arteriography and hepatic arteriography demonstrated no particular abnormalities. Based on these imaging studies, he was diagnosed as having lower bile duct carcinoma and peribiliary cysts. He underwent pylorus preserving pancreatoduodenectomy. As for the peribiliary cysts, a course of observation was decided upon. The carcinoma was a nodal infiltrating-type tumor sized 12 mm × 8 mm existing in the lower bile duct.

The tumor was histopathologically diagnosed as a moderately differentiated tubular adenocarcinoma, pT3, pN0, pM0 and stage IIA[3]. After the operation, minor leakage of the pancreatojejunostomy developed and was successfully treated by conservative therapy. He is alive without recurrence and symptoms 10 mo after surgery at this writing.

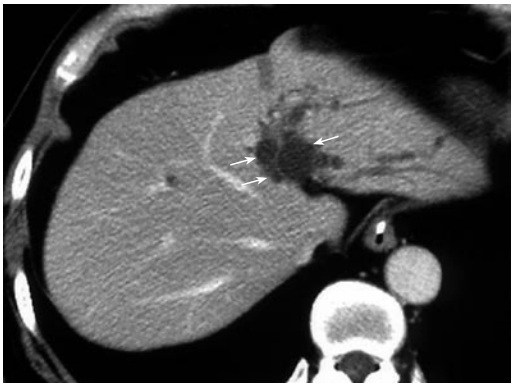

Figure 1 CT revealing multiple small cystic lesions along the umbilical portion of the portal vein (arrows).

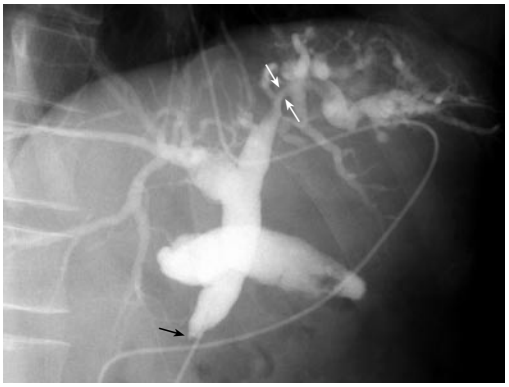

Figure 2 Cholangiogram via endoscopic naso-biliary drainage tube revealing complete obstruction in the lower bile duct (black arrow) and smooth stenosis of the left hepatic duct (white arrows) and a slight dilatation of the intrahepatic bile duct in the left lobe.

DISCUSSION

Peribiliary cysts were first reported by Nakanuma *et al* in 1984[4]. They speculated that the peribiliary cysts arose from cystic dilatation of the intrahepatic extramural peribiliary glands in the periductal connective tissue[4]. A systematic study of autopsied livers disclosed that the disease is not uncommon; cystic changes of the peribiliary glands were found in 202 livers (20.2%) among 1000 consecutive autopsy livers[1]. However, since the degree of cystic dilatation is quite variable, and the majority are recognizable microscopically, this disease is still poorly recognized in clinical practice[5]. The precise mechanism for cystic dilatations of intrahepatic peribiliary glands is unclear. Peribiliary cysts are usually detectable in pre-existing hepatobiliary diseases such as liver cirrhosis, idiopathic portal hypertension, portal vein thrombosis, alcoholic liver disease, and adult-type polycystic kidney disease[4,6,7]. Therefore, it has been speculated that circulation failure caused by the interference of blood flow through the portal vein causes morphological changes of extramural glands around the bile duct. Nakanuma presumed that, in cases of adult-type polycystic kidney disease, gene expression in the intrahepatic peribiliary glands related to this disease may lead to the formation of peribiliary cysts[5]. In the case reported here, alcoholic liver injury did not develop into liver cirrhosis, and there were no findings of portal hypertension. Peribiliary cysts are also known to develop in patients with cystic dilatation of the intrahepatic biliary system after hepatic portoenterostomy[8]. We speculate that in the case reported here, cholestasia due to lower bile duct cancer, in addition to alcoholic liver disease, might be related to the development of peribiliacy cysts. However, whether peribiliary cysts existed before the development of bile duct cancer is uncertain, since he had not undergone diagnostic imaging before the onset of obstructive jaundice.

On diagnostic imaging, peribiliary cysts are characterized as clusters of relatively small multiple cystic lesions developing around the hepatic hilum and large branches of the portal vein, but not in liver parenchyma[9]. Recently, MRCP has been reported to be useful in the diagnosis of peribiliary cysts. MRCP clearly depicts multiple cysts as a string of bead-like structures or a foamy fringe along the hepatic hilum or larger bile ducts. Drip infusion cholangiographic (DIC) CT is also useful for diagnosing peribiliary cysts. On DIC-CT, peribiliary cysts are not filled with contrast material since these cysts do not communicate with the lumen of the biliary tree.

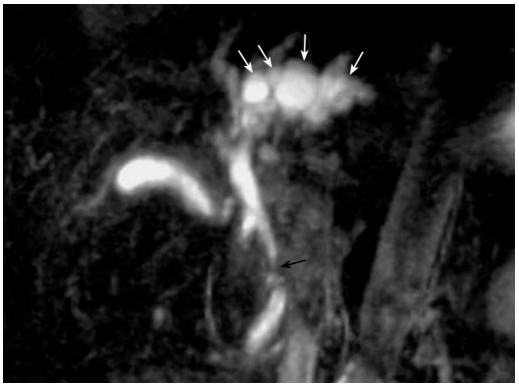

Figure 3 Magnetic resonance cholangiopancreatogram showing bead-like cystic lesions (arrows) along the biliary tree in the left lobe of the liver.

Peribiliary cysts have usually been considered to be clinically harmless. There are two reports describing obstructive jaundice secondary to the obstruction of bile ducts by peribiliary cysts[2,10]. It is thought that surgery is not indicated for asymptomatic peribiliary cysts but they should be followed up[9,11]. Misdiagnosis of peribiliary cysts as intrahepatic cholangiocarcinoma is a possibility[2,12,13], and there are two reports concerning such patients undergoing hepatectomy on suspicion of intrahepatic cholangiocarcinoma[12,13]. Precise diagnosis can be obtained easily by characteristic findings with diagnostic imaging if the attending clinician is knowledgeable about peribiliary cysts. Needless to say, unnecessary operations for patients with peribiliary cysts should be avoided.

There have been no reports of peribiliary cysts accompanying bile duct carcinoma. If knowledge of the clinical entity of peribiliary cysts becomes widespread, the number of patients with peribiliary cysts accompanying biliary malignancy is sure to increase. When peribiliary

cysts and biliary malignancy exist simultaneously, the operative procedure should be chosen according to the staging and extension of the malignancy as well as the positional relationship between the two lesions based on proper diagnosis. We chose not to select surgical therapy for the peribiliary cysts, on the basis that we diagnosed the stricture of the left hepatic duct as compression by peribiliary cysts, and hepatopancreatoduodenectomy, an indispensable procedure for resecting both the lower bile duct cancer and stricture of left hepatic duct, by itself is associated with high rates of postoperative complications and operative mortality[14]. Over-surgery due to misdiagnosis for patients with biliary malignancy accompanied by peribiliary cysts should be avoided. However, peribiliary cysts have been reported to become gradually enlarged and increased in number in some cases[15,16]. Long-term follow-up is obviously called for.

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CASE REPORT

Volvulus of the gall bladder diagnosed by ultrasonography, computed tomography, coronal magnetic resonance imaging and magnetic resonance cholangio-pancreatography

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Abstract

A 54-year-old woman was admitted to our hospital with the complaint of right upper quadrant pain. Upon physical examination the vital signs of the patient were within normal ranges. Ultrasonography and computed tomography (CT) examination of the abdomen was obtained, which demonstrated a large dilatated cystic structure, measuring approximately 68.6 mm × 48.6 mm, with marked distension and inflammation. Additionally, the enhanced CT was characterized by the non-enhanced wall of the gallbladder. As the third examination in this study, magnetic resonance imaging (MRI), namely coronal MRI and magnetic resonance cholangio-pancreatography (MRCP), were performed. The MRCP demonstrated a dilatation of the gallbladder but detected no neck of the gallbladder. Simple cholecystectomy was performed. Macroscopic findings included a distended and gangrenous gallbladder, and closer examination revealed a counterclockwise torsion of 360 degrees on the gallbladder mesentery. Coronal MRI and MRCP showing characteristic radiography may be useful in making a definitive diagnosis.

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Key words: Volvulus of the gallbladder; Computed tomography; Magnetic resonance imaging; Magnetic resonance cholangio-pancreatography

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INTRODUCTION

Gallbladder volvulus is a relatively rare disease and well recognized in the elderly people. It has been reported in only about 300 cases in the literature ranging in age from 2 to 100 years old. Preoperative diagnosis of gallbladder volvulus has always been considered difficult. Although recent advances in radiographic finding have helped in the diagnosis of many diseases, abdominal computed tomography (CT) and Ultrasonography (US) remain nonspecific in diagnosing volvulus of the gallbladder. However, we could make definitive radiographic imaging by coronal magnetic resonance imaging (MRI) and magnetic resonance cholangio-pancreatography (MRCP).

CASE REPORT

A 54-year-old woman was admitted to our hospital with the complaint of right upper quadrant pain of approximately 5 hours in duration. Pain was accompanied by anorexia and nausea without vomiting and was not preceded by jaundice. She had no relevant past history. Upon physical examination the vital signs of the patient were within normal ranges. Right upper quadrant tenderness and Murphy's sign were detected in the abdominal examination. Laboratory data showed a leukocyte count of 11 800/mm^3 and C-reactive protein (CRP) of 2.57 mg/dL. Initial treatment consisted of administering intravenous fluids and broad-spectrum antibiotics. Abdominal ultrasound US demonstrated a distended, fluid-filled neck of abnormal swelling, a normal-walled gallbladder with surrounding ascites and edema (Figure 1), but no stones. Secondary

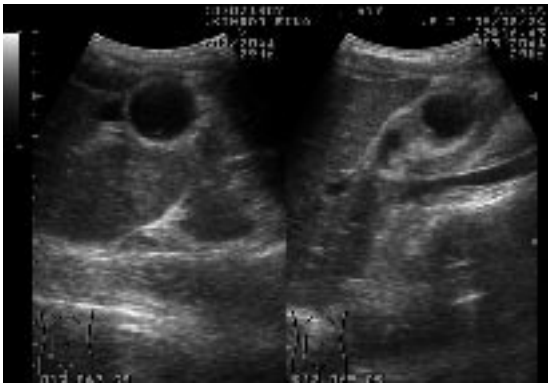

Figure 1 Abdominal ultrasonography revealed an abnormally large floating gallbladder without gallstones, and a thickened gallbladder wall.

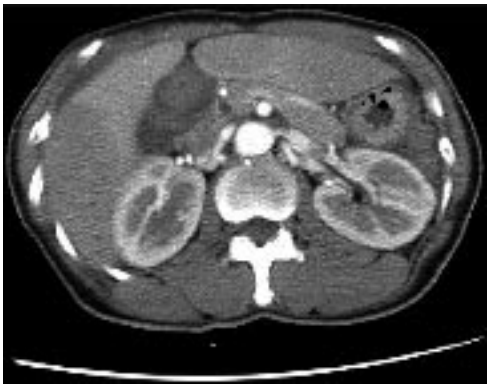

Figure 2 Abdominal enhanced computed tomography revealed a dilatated gallbladder, but a non-enhanced gallbladder wall.

computed tomography (CT) examination of the abdomen was obtained. CT demonstrated a large dilatated cystic structure, measuring approximately 68.6 mm × 48.6 mm, with marked distension and inflammation. Additionally, the enhanced CT was characterized by the non-enhanced wall of the gallbladder (Figure 2). As the third examination in this study, magnetic resonance imaging (MRI), namely coronal MRI and magnetic resonance cholangiopancreatography (MRCP), were performed. The MRCP demonstrated a dilatation of the gallbladder but detected no neck of the gallbladder (Figure 3). The coronal MRI revealed a dilatated gallbladder, and additionally an imagination that identified the neck of the gallbladder (Figure 4). She was diagnosed with acute cholecystitis and volvulus of the gallbladder according to the findings of US, CT, MRI and MRCP and underwent percutaneous transhepatic gallbladder aspiration (PTGBA) for one day. Following the treatment, a hemobilia discharge from the PTGBA was noted, and she was diagnosed with necrotic gallbladder. Her pain was slightly improved, but she still had tenderness and high fever. She underwent open laparotomy, and simple cholecystectomy was performed. Macroscopic findings included a distended and gangrenous gallbladder, and closer examination revealed a counterclockwise torsion of 360 degrees on the gallbladder mesentery. De-torsion and cholecystectomy were easily performed (Figure 5). The histopathology report showed

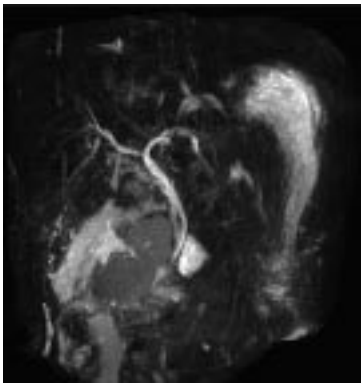

Figure 3 MRCP demonstrated dilatation of the gallbladder, but its image identified no gallbladder neck.

Figure 4 Coronal MRI revealed a dilatated gallbladder, and its invagination-like image identified the neck of the gallbladder.

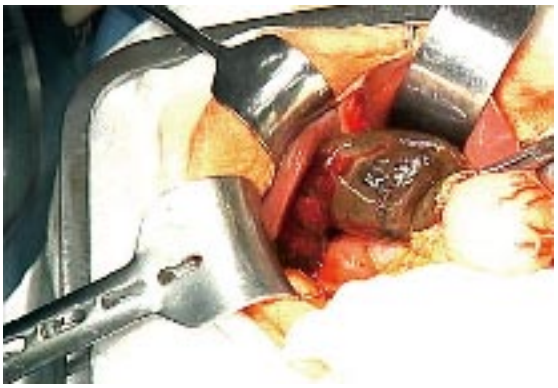

Figure 5 At laparotomy, macroscopic findings showed a distended and gangrenous gallbladder along with a counterclockwise torsion of 360 degrees of the gallbladder mesentery.

necrosis and hemorrhage of the gallbladder without evidence of lithiasis. Nine days after the operation she was discharged with no complications.

DISCUSSION

First reported by Wendel in 1898[1], volvulus of the gallbladder is a relatively uncommon phenomenon, with no more than 300 cases reported in the literature. It occurs in all age groups, with the highest incidence in elderly women, and a female-to-male ratio of 3:1. Perhaps the incidence would increase with a longer life expectancy rate[2,3]. Gallstones are unlikely to be the cause of gallbladder torsion, as gallstones are not uniformly present in all patients reported with torsion. One study of

245 patients found stones in only 24.4%; 51% developed a clockwise torsion rotation[4]. Although supportive evidence is lacking, inferences have been made in the literature linking gastric peristalsis to clockwise gallbladder torsion and colonic peristalsis to counterclockwise torsion[5]. Because volvulus of the gallbladder is a relatively uncommon phenomenon, preoperative diagnosis of gallbladder torsion remains difficult. Therefore, most cases are diagnosed intraoperatively at present. Also, laboratory evaluations are often nonspecific. For example, white blood cell count (WBC), CRP and creatine phosphokinase (CPK) are frequently elevated, while liver function tests are commonly normal. In our case, WBC, CRP and CPK were elevated. Although recent advances in radiographic studies have helped in the diagnosis of many diseases, radiographic studies remain nonspecific in diagnosing volvulus of the gallbladder. Fewer than a dozen cases have been reported in the literature where a preoperative diagnosis was made. Ultrasound studies often reveal a large floating gallbladder without gallstones, and a thickened gallbladder wall. Specific ultrasound signs seen with gallbladder torsion include the presence of the gallbladder outside its normal anatomic fossa, inferior to the liver or in a transverse orientation with an echogenic conical structure[6]. Additionally, CT findings are also nonspecific. A few cases of CT diagnosis of gallbladder torsion commented on radiographic findings of marked enlargement of the gallbladder with an unusual shape and configuration[7,8]. MRI has been used to establish a diagnosis preoperatively. MRI findings include high signal intensity within the gallbladder wall on T1 weighted images, suggesting necrosis and hemorrhage consistent with gallbladder torsion. In the present case, MRCP revealed anatomic details of the neck of the gallbladder and cystic duct.

We consider the characteristics of these radiography images to be useful for differential diagnosis of torsion of the gallbladder from gallbladder stone and gallbladder cancer. Usui *et al* have reported that only MRCP made it possible to determine the relationships between the distorted bile ducts, the interrupted cystic duct, and the enlarged gallbladder, and it was a relatively non-invasive procedure[9]. In addition, Shaikh *et al* have reported that the presence of a redundant mesentry was a prerequisite for torsion[10]. Although torsion of the gallbladder is a rare occurrence, the diagnosis should be considered in all patients presenting with right upper quadrant pain. If diagnosed early and treated, it remains a benign condition; however, a delay in diagnosis and management may lead to sequelae associated with gallbladder rupture and biliary peritonitis. US, CT, and magnetic resonance techniques, especially coronal MRI and MRCP, are useful in diagnosing volvulus of the gallbladder. About 300 cases have been described in the literature so far, but only a minor portion (putatively less than 50 cases) have preoperative imaging studies such as CT and MRI. Ours is the first case in the medical literature in English to report on volvulus of the gallbladder diagnosed by coronal MRI and MRCP showing characteristic radiography.

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CASE REPORT

Branch retinal vein thrombosis and visual loss probably associated with pegylated interferon therapy of chronic hepatitis C

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Abstract

Ophthalmological complications with interferon therapy are usually mild and reversible, not requiring the withdrawal of the treatment. We report a case of a patient who had visual loss probably associated with interferon therapy. Chronic hepatitis C virus infection (genotype 1a) was diagnosed in a 33-year old asymptomatic man. His past medical history was unremarkable and previous routine ophthalmologic check-up was normal. Pegylated interferon alpha and ribavirin were started. Three weeks later he reported painless reduction of vision. Ophthalmologic examination showed extensive intraretinal hemorrhages and cotton-wool spots, associated with inferior branch retinal vein thrombosis. Antiviral therapy was immediately discontinued, but one year later he persists with severely decreased visual acuity. This case illustrates the possibility of unpredictable and severe complications during pegylated interferon therapy.

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Key words: Hepatitis C; Interferon; Visual loss

Gonçalves LL, Farias AQ, Gonçalves PL, D'Amico EA, Carrilho FJ. Branch retinal vein thrombosis and visual loss probably associated with pegylated interferon therapy of chronic hepatitis C. *World J Gastroenterol* 2006; 12(28): 4602-4603

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INTRODUCTION

Pegylated interferon has been shown to be associated with high rates of sustained virological responses and has become the standard therapy of chronic hepatitis C. However, interferon therapy may be associated with severe adverse effects. We report the case of a patient who developed retinal vein thrombosis and visual loss probably related to the antiviral therapy.

CASE REPORT

A 33-year old asymptomatic Caucasian man was referred for investigation of abnormal liver enzymes. Chronic hepatitis C virus infection, genotype 1a, with viral load 220 400 IU/mL, was diagnosed, and a liver biopsy showed chronic hepatitis, with inflammatory activity A2 and fibrosis F2 according to the METAVIR system. Past medical history was unremarkable and negative for diabetes mellitus, arterial hypertension, dislipidemia, obesity, regular use of medications, smoking and illicit drug or alcohol abuse. Previous routine ophthalmologic check-up was normal, with a visual acuity 20/20. Pegylated interferon α 2b was started at the dose of 1.5 μg/kg once weekly in combination with ribavirin 1000 mg/d. Three weeks later he reported painless blurring of vision. The ophthalmologic examination confirmed the reduction of the visual acuity and showed extensive intraretinal hemorrhages and cotton-wool spots, associated with inferior branch retinal vein thrombosis. Fluorescein angiography confirmed the venous occlusion (Figure 1). The antiviral therapy was immediately discontinued. Hematocrit, platelet count, glucose, prothrombin activity, creatinine, cholesterol and triglycerides levels were normal. Activated protein C resistance phenotype was normal. The levels of protein S, antithrombin Ⅲ, homocysteine and fibrinogen were within the normal ranges. Plasma protein C was 50% (normal 58%-125%), compatible with heterozygous state. Cryoglobulins and antiphospholipids antibodies were negative. Factor V Q 506 and prothrombin gene 3'-UTR G20210A mutant alleles were absent. Doppler ultrasound of the heart and carotid arteries was normal. The patient developed macular edema which required panretinal photocoagulation. One year later, he persists with severely decreased visual acuity.

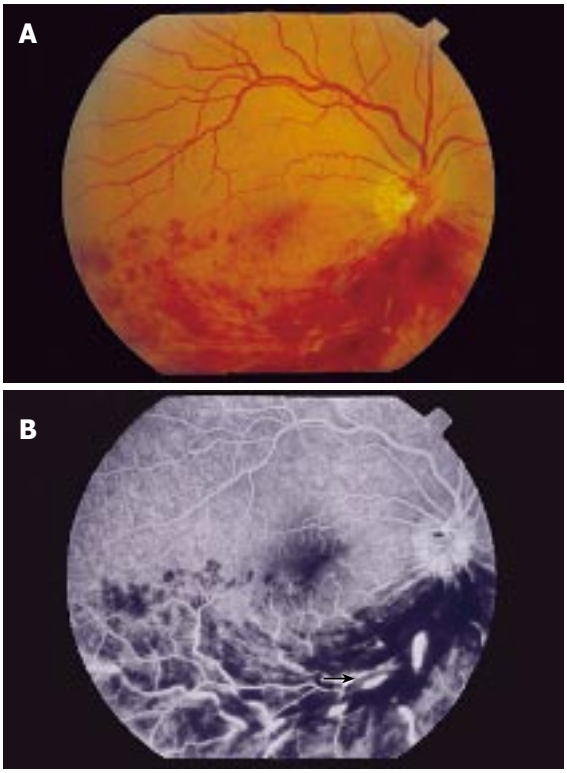

Figure 1 **A:** Fundus photograph of the right eye with extensive intraretinal hemorrhages and cotton-wool spots in the inferonasal and inferotemporal regions; **B:** Fluorescein angiography with segmental hypoperfusion, dilation and tortuosity of the retinal veins (arrow), compatible with inferior branch retinal vein thrombosis.

DISCUSSION

Ophthalmologic complications with interferon alpha therapy, such as retinopathy with cotton-wool spots, hemorrhages and macular edema, optic neuropathy and thrombotic microangiopathy, occur in less than 1% of treated patients. Individuals with diabetes, hypertension, dislipidemia and hypercoagulable states are more prone to develop those changes. In most cases they are subclinical, mild and reversible, not requiring the withdrawal of the treatment.

The physiopathology of retinal vein thrombosis in patients with viral hepatitis is poorly understood, but may be related to both background predisposition and side effects of therapy. Protein C deficiency occurs in 1 of 250 controls and leads to impaired inhibition of clot formation, which is associated with an increased risk for venous thrombosis (relative risk, 7.3; annual incidence, 1%)[1]. However, the frequency of venous thrombosis in protein C deficiency state is highly variable. Only a minority of affected individuals develop thrombosis, suggesting that the presence of another simultaneous risk factor is needed.

Both chronic hepatitis C and interferon therapy are associated with the formation of procoagulant antibodies, in particular antiphospholipids antibodies, which may predispose to thrombosis. In addition, interferon therapy induces the increase of plasma-activated complement C5a, a potent intravascular aggregator of granulocytes, favoring the development of microthrombi in small vessels[2,3].

The patient developed a severe, early and sight losing complication of antiviral therapy. Most reported cases occurred in predisposed individuals 2 to 11 months after the beginning of standard alpha interferon therapy. However, one could speculate that interferon pegylation could modify both the pharmacokinetics of the drug and the timing of retinal vein thrombosis.

Since there are few reported cases of retinal vein thrombosis[3-5], this case may represent a coincidence, not associated with interferon therapy. Nevertheless, it is noteworthy that the patient was asymptomatic and had a normal vision before antiviral therapy. Although current guidelines of therapy of chronic viral hepatitis do not support routine ophthalmologic screening and work-up for hypercoagulable states in all patients treated with interferon, this case illustrates the possibility of unpredictable and severe complications during pegylated interferon therapy.

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
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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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

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
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
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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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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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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]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, ρ (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2 not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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