

Extraintestinal manifestations in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD) can be really considered to be systemic diseases since they are often associated with extraintestinal manifestations, complications, and other autoimmune disorders. Indeed, physicians who care for patients with ulcerative colitis and Crohn's disease, the two major forms of IBD, face a new clinical challenge every day, worsened by the very frequent rate of extraintestinal complications. The goal of this review is to provide an overview and an update on the extraintestinal complications occurring in IBD. Indeed, this paper highlights how virtually almost every organ system can be involved, principally eyes, skin, joints, kidneys, liver and biliary tracts, and vasculature (or vascular system) are the most common sites of systemic IBD and their involvement is dependent on different mechanisms.

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INTRODUCTION

Inflammatory bowel diseases (IBD) can be really considered to be systemic diseases since they are often associated with extraintestinal manifestations, complications, and other autoimmune disorders. Indeed, physicians who care for patients with ulcerative colitis (UC) and Crohn's disease

(CD), the two major forms of IBD, face a new clinical challenge every day, worsened by the very frequent rate of extraintestinal complications. Virtually almost every system can be involved, principally eyes, skin, joints, kidneys, liver and biliary tracts, and vasculature (or vascular system) are the most common sites of systemic IBD and their involvement is dependent on different mechanisms.

Extraintestinal IBD-related immune disease can be classified into two major groups: the first one includes reactive manifestations often associated with intestinal inflammatory activity and therefore reflecting a pathogenic mechanism common with intestinal disease (arthritis, erythema nodosum, pyoderma gangrenosum, aphthous stomatitis, iritis/uveitis)^[1,2] (Table 1); the second one includes many autoimmune diseases independent of the bowel disease that reflect only a major susceptibility to autoimmunity. They are not considered (apart for primary sclerosing cholangitis) as specific IBD features but only as autoimmune associated diseases such as ankylosing spondylitis, primary biliary cirrhosis, alopecia areata, and thyroid autoimmune disease and others^[1] (Table 2).

Moreover, many extraintestinal complications due to metabolic or anatomical abnormalities caused directly by

Table 1 Major extraintestinal immune-related manifestations of IBD

Arthritis
Erythema nodosum
Pyoderma gangrenosum
Aphthous stomatitis
Iritis/uveitis

Table 2 Autoimmune disorders associated to IBD

Alopecia areata
Ankylosing spondylitis
Bronchiolitis obliterans
Cold urticaria
Hemolytic anemia
Henoch-Schoenlein purpura
Insulin-dependent diabetes mellitus
Pancreatitis
Primary biliary cirrhosis
Primary sclerosing cholangitis
Polymyositis
Raynaud phenomenon
Seropositive rheumatoid arthritis
Sjogren syndrome
Thyroid disease
Vitiligo
Wegener's granulomatosis
Takayasu's arteritis

Table 3 Extraintestinal complications in IBD and principal pathogenetic mechanisms of arthritis

Extraintestinal complications in IBD	Principal pathogenetic mechanisms of arthritis
Anemia	Iron deficiency, inflammation
Thromboembolic events	Hypercoagulopathies, platelet activation
Osteopathy	Steroid therapy, vitamin D deficiency inflammation
Growth failure	Malnutrition
Nephrolithiasis	Dehydration, hyperoxaluria, low urinary PH
Cholelithiasis	Intestinal loss of bile acids
Amyloidosis	Acute phase reaction, chronic inflammation
Fatty liver	Malnutrition

IBD have been reported frequently and include osteoporosis, biliary and urinary lithiasis, and anemia (Table 3).

Aim of this paper is to review the pathogenic mechanisms, frequency, features, and therapy of the major IBD-associated extraintestinal manifestations.

Pathogenesis of immune-related extraintestinal manifestation in IBD

Extraintestinal immune-related manifestations in IBD are directly dependent on intestinal disease, often coexist in the same patients and have probably the same, even if not completely clarified, pathogenesis^[2]. Evidence coming from many studies in genetically susceptible animal models of colitis suggests the crucial role of enteric flora in activating the immune system against bacterial antigens and contemporary against colonic mucosa on the basis of an antigenic cross-reactivity ("antigen mimicry")^[3]. The sharing of these colonic antigens by extraintestinal organs, associated with a genetic susceptibility, would finally lead to an immune attack to these organs^[2]. One of the best example is represented by primary sclerosing cholangitis occurring in UC: in a subset of patients, the presence (in sera and colonic mucosa) of anti-colonic mucosa auto-antibodies that cross react with biliary epithelium has been identified^[4]. Furthermore, recently a colonic epithelial protein (CEP) and the human tropomyosin isoform 5 (hTM5), which are not only expressed in the colon but also in the biliary tract, skin, eyes, and joints, have been suspected to be the major common targets of autoimmune attack in extraintestinal organs of IBD patients being IgG1 specific auto-antibodies identified in UC patients presenting multiple extraintestinal manifestations^[5].

It remains unclear why the extraintestinal organs are not always involved at the same time and why these auto-antibodies are absent in colonic CD. A partial explanation is that genetic factors or local co-existent damage factors (infections, trauma) could regulate the display of cryptic antigens and the susceptibility to autoimmune attack^[2].

According to the previously explained mechanism, we can identify an immune induction site, where T cells are primed, represented by the colon and the effectors sites that are the extraintestinal organs. Immune cells infiltrate the effectors sites (where they will proliferate) with the help of adhesion molecules ($\alpha 4\beta 7$ integrin, vascular adhesion protein 1) that have a cytokine-mediated

overexpression in specific tissues^[6].

It is interesting how autoimmune attack can happen many years after the removal of the colon. In the case of primary sclerosing cholangitis (PSC), probably memory lymphocytes that have been primed in the bowel can recirculate for many years also even after the removal of the colon without causing damage until the occurrence of a stimulus in the liver that activates inflammation with the overexpression of adhesion molecules (MAdCAM and CCL 25) and consequent persistent lymphocytes recruitment^[7]. Interference with adhesion molecules could be useful in the treatment of extraintestinal manifestation as it has already been shown for the intestinal inflammatory activity^[8].

Genetic susceptibility

Extraintestinal manifestations have certainly a familial predisposition (83% of concordance between siblings)^[9] and this suggests the existence of a strong genetic influence leading to the identification of many suspected predisposal genes.

HLA system is considered as one of the major genetic markers associated with IBD and extraintestinal manifestations, probably a specific and appropriate antigen presentation that leads to autoimmune reaction in particular predisposing conditions.

It has been reported that UC patients who display HLA-B8, DR3 phenotype have a 10-fold higher risk of primary sclerosing cholangitis^[10].

Moreover, UC patients who have HLA DRB1*0103 (DR103) have a higher risk of ocular and articular manifestations^[11] and patients with HLA-B*27 and B*58 have a higher risk of uveitis (Orchard TR 2002). HLA-B*27 is strongly associated with ankylosing spondylitis (AS) being present in 90% of these patients but it seems not to be significantly associated with IBD; anyway IBD patients with HLA-B*27 positiveness have a higher risk to develop AS and IBD patients with axial articular involvement are HLA-B*27 positive from 25% to 75%^[12]. The polymorphism -1031 C TNF- α has been associated with erythema nodosum in IBD patients^[13]. It is possible that HLA genes interfere actively in the pathogenesis of IBD-extraintestinal manifestations or that are in linkage disequilibrium with other really responsible unknown genes. Moreover, caspase-activation recruitment domain containing protein 15 (CARD15), a gene found in association with ileocecal CD with a potential role in the bacterial handling, has been recently associated with sacroiliitis even if previous results did not agree^[14].

Joint involvement in IBD

Inflammatory arthropathies are the most common extraintestinal manifestations in IBD patients with a prevalence ranging between 7% and 25%^[1,12]. Articular and musculoskeletal manifestations are included in the spondyloarthropathies (SpAs) that are a group of seronegative autoimmune related disorders with common characteristics including: ankylosing spondylitis, reactive arthritis, psoriatic arthritis, inflammatory bowel disease,

some forms of juvenile arthritis and acute anterior uveitis^[15].

Articular involvement (peripheral or axial) can precede, be synchronous or begin afterward the diagnosis of IBD, it is characteristically pauciarticular, asymmetrical, transitory, migrating, prevalently non deforming. The axial involvement can vary from asymptomatic sacroiliitis to inflammatory lower back pain to ankylosing spondylitis (that occurs in 3% of IBD patients)^[1].

It is interesting that the high incidence of asymptomatic sacroiliitis (varying from 10% to 52%)^[16,17,18] and on the other hand the equally high incidence (about 50%) of characteristic inflammatory low back pain in the absence of radiological findings in IBD patients^[12] indicating how history and physical examination should be the diagnostic tools. Peripheral arthritis, different from axial involvement, has a significant positive association with the skin, mouth, and ocular manifestation; it happens more frequently in CD (and particularly in colonic localization), often accompanies intestinal activity ameliorating with IBD pharmacological or surgical treatment^[1]. In some patients, despite the amelioration of gut inflammatory activity, articular disease persists^[19].

Arthritis is often associated with enthesitis, tenosynovitis, dactylitis that can also appear in the absence of arthritis^[20]; typically they do not alter inflammatory markers and can compromise deeply the quality of life. Conventional treatment of inflamed joints include nonsteroidal anti-inflammatory drugs and cyclooxygenase-2-inhibitor that should be used for short-term period because of gastrointestinal side effect and IBD reactivation risk^[21,22] nevertheless at drug suspension articular relapse can occur. Also local intra-articular steroid treatment can be useful.

The majority of interventional studies included undifferentiated spondyloarthropathies- or ankylosing spondylitis patients and no IBD patients, so that the results can only be extrapolated. Sulfasalazine has been shown to be effective in peripheral joint disease in SpAs patients^[23,24] and positive but limited results have been obtained with methotrexate^[25].

Interesting results have been obtained with anti TNF- α inhibitors in resistant SpAs with IBD. In uncontrolled studies, infliximab has shown efficacy in the treatment of SpAs in CD patients as induction and maintenance therapy^[26-28], also in the absence of acute phase reactants and intestinal activity; there is also a reported case of positive results in SpA associated with ulcerative colitis^[29]. In limited reports (two cases) etanercept, that is ineffective in IBD treatment^[30], has given good results in the treatment of SpAs associated to CD^[31]. It is not clear at the moment the effect of anti TNF- α therapies on articular damage evolution, certainly the early recognition and appropriate treatment can help to limit the patient's inability.

Hepatobiliary disease

Hepatobiliary diseases are common in IBD patients; they can or cannot be immuno-mediated and can also depend

on side effects of medications (see Table 4). Elevation of liver function tests have been observed from 11% to 49% in IBD patients in observational studies^[32-34]. The most common immuno-mediated hepatobiliary disease is primary sclerosing cholangitis (PSC) that is a chronic cholestatic disorder characterized by inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts. It is more frequent in male individuals, and the prevalence of IBD (mostly UC) in PSC is about 70-80%^[35]. Conversely about 2-7% of UC patients^[35,1] and 0.7-3.4% of MC patients have a diagnosis of PSC^[36,1]. Suggestive symptoms of PSC are fatigue, pruritus, jaundice, and abdominal discomfort but it is not rare that the isolate finding of abnormalities in liver biochemical markers (first of all alkaline phosphatase); in fact 15-70% of PSC patients are asymptomatic^[37,1]. There are no specific auto-antibodies and so biopsy or cholangiography is often necessary for the diagnosis. In PSC patients, IBD frequently present some specific features: pancolonic extension with rectal sparing, backwash ileitis, low intestinal activity, and high pouchitis incidence after colectomy. These distinguishing features have suggested the existence of an IBD-PSC specific clinical phenotype^[38]. It is well-known that the increased risk of colonic dysplasia/carcinoma in PSC patients compared to the general population (10-fold risk)^[39] and to other UC patients^[35] it could depend on the long-lasting and asymptomatic colitis (consequently often underestimated) and by alterations in bile salts pool or folate deficiency. Similarly it has significantly increased the risk of bile duct cancer^[40] and metabolic bone disease^[41,38]. PSC has a median survival time of 9-12 years from the time of diagnosis; it seems that neither concomitant IBD presence nor colectomy (in UC patients) alter its natural history. Moreover, the colorectal cancer risk does not seem to be decreased after liver transplantation^[42].

The therapeutic possibilities in PSC are limited; the best results have been obtained with UDCA at a high dose (until 20 mg/kg)^[43-45] and the combined use of corticosteroids showed only a little additive benefit^[46,47].

The best clinical approaches to PSC include colonoscopic examination with a biopsy to identify a possible asymptomatic UC and/or cancer and a prevention colonoscopy program in patients identified to have had UC. Association between primary biliary cirrhosis (PBC) and UC is rare but possible and it has been reported in 15 cases; similarly to the CSP it seems that colectomy does not alter the progression of the hepatic disease^[48].

Apart from classical immunological liver diseases in IBD patients, they are often observed for other abnormalities. Liver enlargement is the most common reported finding and is strictly related to steatosis grade. Steatosis has been described in more than 30% of patients and it does not seem to be related to the kind of IBD and sex. Data about the influence of disease activity and pharmacological treatment on steatosis are contradictory^[49].

Also cholelithiasis is more frequent in IBD patients (about 10%) than in the general population (7%) and mainly in CD (first of all in ileal localization); it seems

to correlate with female sex, previous surgery (mainly ileal resection), and old age. In limited series, it has been shown to be less often symptomatic than in the general population^[49]. Probably cholelithiasis is caused by bile salt pool alteration for malabsorption.

Rare complications reported in literature are liver abscesses; it is thought that in most cases portal bacteremia, favorite by mucosal barrier alterations, can be the principal mechanism; rarely an ascending acute cholangitis in PSC has been suspected to be the cause^[50]. Portal vein thrombosis and suppurative pylephlebitis has also been described in rare cases^[49].

Cutaneous manifestations

Cutaneous manifestations of IBD are relatively common. The incidence varies from about 10% at the time of IBD diagnosis to more than 20% in the course of the disease^[1]. Skin lesions can be classified into three principal classes: granulomatous, reactive, and secondary to nutritional deficiency.

Granulomatous cutaneous lesions have the same histological features of the bowel disease and include: perianal and peristomal ulcers and fistulas, metastatic CD, oral granulomatous ulcers.

Perianal disease is very frequent occurring in about 50% of CD patients during their clinical history, and it varies from perianal erythema to abscesses and perianal complex fistulae^[51].

Other fistulae can be internal or entero-cutaneous; rarely develop on the abdominal scar of laparotomy or at the umbilicus. Many efforts have been made to treat fistulizing disease, and the classical surgical approach has been supported recently by a larger use of drugs. Antibiotics, azathioprine/mercaptopurine, tacrolimus, thalidomide showed efficacy in uncontrolled studies^[52] but at the moment only infliximab showed effectiveness as induction^[53] and maintenance therapy^[54] in phase III controlled trials; consequently it has been approved as first line therapy in perianal and enterocutaneous fistulizing CD by the United States Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products in Europe. Surgical procedures as colostomy in the more severe cases of perianal fistulas, fistulotomy, abscesses drainage, and non-cutting setons placement seem to be very useful, and the combined medical and surgical approach is probably the best one^[55].

Metastatic CD is a rare complication defined as the occurrence of specific granulomatous cutaneous lesions remote from the intestinal disease^[56]. It manifests as subcutaneous nodules or ulcers mainly at the lower extremities with rare case of genital (testicular and vulvar) localizations. It seems unrelated to the bowel activity. Corticosteroids, antibiotics, azathioprine, methotrexate^[57], and more recently infliximab^[58,59] have been used successfully.

The group of reactive skin manifestations of IBD includes aphthous stomatitis, erythema nodosum, pyoderma gangrenosum, and the rare Sweet's syndrome.

Aphthous stomatitis is observed in about 10% of

patients: it occurs generally during active intestinal disease, often recurs and shows a good response to intestinal treatment.

The prevalence of erythema nodosum in IBD is 3-8%. It appears more often in women, in the colonic localization, in concomitance with arthritis and active intestinal disease^[1]; furthermore, there is a positive response to proctocolectomy^[42].

Histological examination shows lympho-histiocytic infiltrate of the lower derma. On the basis of uncontrolled data, corticosteroids are generally an effective therapy; also immunosuppressive therapy is used^[56]. Resistant cases have been treated effectively with infliximab^[60].

Pyoderma gangrenosum is a very debilitating ulcerating chronic skin disorder occurring in about 1-2% of IBD patients. It occurs often on the extensor surface of the legs, particularly in coincidence with exacerbation of intestinal disease and in association with other extraintestinal manifestations (arthritis and erythema nodosum)^[1,61]. Moreover, it is often associated with colonic involvement and in UC patients it seems to benefit lesser than erythema nodosum from colectomy^[42].

According to disease severity, the treatment (based on non-controlled evidence) can be local or systemic and includes a high dose of oral or intralesional corticosteroids, immunosuppressive/immunomodulatory therapy (cyclosporine, tacrolimus, mycophenolate mofetil, azathioprine, dapsone)^[61]. Infliximab has shown to be very effective in the refractory disease and can be used as first choice therapy^[59,62,63]; also etanercept has been reported to be effective in a refractory case of pyoderma gangrenosum^[64].

Another rare cutaneous manifestation associated with IBD is the Sweet's syndrome. It is a neutrophilic dermatosis probably related to pyoderma gangrenosum consistent with painful erythematous plaques or nodules often associated with fever and leukocytosis that usually responds to corticosteroids^[65].

The more frequent nutritional-deficient cutaneous manifestation is the acrodermatitis enteropathica; it is caused by zinc deficiency and manifests as a psoriasis form erythema^[61].

Furthermore, an association between autoimmune cutaneous disease and IBD has been reported. The most frequent disease is psoriasis (7-11% of IBD population *vs* 1-2% of general population) than vitiligo and more rarely are polymyositis, lupus erythematosus and scleroderma^[66].

Ocular manifestations of IBD

Ocular manifestations occur in about 10% of IBD patients. They can be immune-related (episcleritis, scleritis, uveitis, corneal disease) or related to drug exposure (cataract, glaucoma).

Episcleritis manifests as acute redness, irritation, burning, tender to palpation; if there is also an impairment of vision, the presence of a scleritis is possible. In this case, a referral to an ophthalmologist is mandatory for risk of vision loss. Uveitis can be anterior and posterior and is often associated with joints and skin manifestations.

Anterior uveitis is the most common and presents painful eye, visual blurring, and photophobia but can be also asymptomatic and sometimes precedes the diagnosis of IBD. Concomitance with IBD activity is typical for episcleritis and uveitis^[1,67].

Corneal disease (potential cause of perforation) has also been reported; conjunctivitis is frequent in IBD population but probably does not differ in frequency and etiologic factors from the general population. Anyway it is important that the clinician is aware that serious ocular disease can mimic conjunctivitis in IBD patients^[67].

Ocular manifestations can benefit first of all by the treatment of the underlying IBD (particularly for anterior uveitis and episcleritis).

Treatment of ocular disease can prevent complications such as retinal detachment or optic nerve swelling in scleritis and secondary glaucoma and cataracts in uveitis.

Cycloplegics, NSAIDs, topical and systemic steroids are useful. Immunosuppression can be necessary in case of scleritis; sulfasalazine/mesalazine seems to prevent anterior uveitis recurrence^[67]. Recently infliximab has shown efficacy in acute uveitis, in episcleritis, and scleritis^[68,69]. Other rare reported ocular manifestations of IBD are: retinitis, orbital IBD, retinal arterial and venal occlusion, optic neuritis, retinal vasculitis, marginal corneal disease, lid margin ulcers^[70,67].

Metabolic osteopathy

IBD is also associated with an increased risk of osteoporosis and osteopenia. The prevalence rates ranges from 2% to 30% for osteoporosis and from 40% to 50% for osteopenia^[71]. The risk of fractures in IBD patients varies widely in different studies^[72-74] (from an OR of 1.41 to 2.5) the real impact being completely clear on the IBD population; because of contrasting results, it is also uncertain if the risk is comparable for UC and MC and for men and women.

Osteoporosis occurrence is often underestimated as shown in an observational study conducted in the UK^[74] in which women aged 65 years with severe IBD have shown a 10-years probability of hip fracture of 7%; nevertheless only 13% of patients who had already sustained a fracture were in bone-sparing therapy.

Furthermore, a significant number of fractures in IBD patients (as in the general osteoporotic population) are asymptomatic (14.2% in a study of Stockbrugger) and many fractures will be underreported^[75].

Bone loss and consequent fractures are certainly multifactorial processes. They are significantly dependent on the age (above 60 years), the use of corticosteroids^[74-76] and the grade of systemic inflammation (intestinal disease activity correlates with the risk of fracture)^[74].

Recently the role of the inflammatory-induced osteopenia has been reevaluated and a surface receptor (RANK) localized on osteoclasts that induces osteoclastogenesis has been identified. Its ligand (RANKL) is induced by proinflammatory cytokines; its decoy receptor that prevents ligation of RANKL to RANK is called osteoprotegerin (OPG), and is produced by osteoblasts and prevents

bone loss. Its production is inhibited by corticosteroids and increased by bisphosphonates. So OPG-RANKL-RANK system is certainly a pivot in inflammatory-induced bone loss^[77,78]. Initial therapeutical use of recombinant OPG in inflammation-induced osteoporosis seems to be promising^[79].

On the basis of these findings, the role of nutritional deficiency could be smaller than previously thought, as also shown in a preliminary recent observational study that found low intake of calcium (<1 000 mg/die) and vitamin D (<200 IU/die) in premenopausal IBD women not to be a predictor of bone loss^[80].

Other factors that could favor osteoporosis are the use of corticosteroids, the hypogonadism, and the immobility. Also genetic markers have been proposed as determinants of bone loss in IBD patients. In the future, they could contribute to identify high-risk patients and support clinical behavior^[81].

Definition of a correct clinical approach is difficult because therapeutic trials on IBD patients with the specific end-point of fractures prevention are lacking and an extrapolation by the major clinical trials on osteoporosis is difficult, since these studies involve postmenopausal patients, certainly older than IBD patients with osteoporosis.

A small randomized, placebo-controlled trial showed that alendronate significantly ameliorates spine bone density compared with the control group after 1-year-long therapy in IBD patients^[82]. Also azathioprine, effective on intestinal activity, seems to have positive effect on bone loss retardation^[83].

The expert recommendations on therapy, in the absence of more specific evidence, do not significantly differ from that of the general population. Supplementation of vitamin D for patients above 60 years, therapy with bisphosphonates in case of osteoporosis (identified with densitometry), osteoporotic fractures and chronically steroid treatment, use of minimum dosage of corticosteroids (preferentially non-systemic), correction of early menopause or male hypogonadism by hormone therapy have also been reported^[84,85]. Recently apart from OPG use, another osteoanabolic substance, the parathyroid hormone 1-34, is under evaluation for the steroid-induced osteoporosis^[86]; in the future they could show effectiveness in IBD osteoporosis. More efforts are yet to be taken in the identification of high-risk patients and in the definition of the most cost-effective clinical behavior in IBD patients.

Thromboembolism and IBD

Patients with IBD have a well-known increased risk (threefold higher than in controls) of thromboembolism (TE), which is an important cause of morbidity and mortality. The incidence ranges from 1.2% to 6.1% according to different studies and in necropsy studies it reaches 39%^[87].

Thrombosis accidents occur prevalently as deep vein thrombosis and pulmonary thromboembolism; they happen in earlier age than in non-IBD patients and are more frequent in active or complicated IBD; the type of

IBD and the sex seems not to influence thromboembolic risk^[87,88]. Using the logistic regression model, it has been found that IBD is an independent risk factor for thrombosis that is a specific IBD feature. In fact, other inflammatory chronic condition as rheumatoid arthritis or chronic intestinal malabsorptive conditions as celiac disease do not show an increased risk of TE^[87].

IBD patients have a frequent exposure to classical thrombosis risk factors: immobility, surgery, steroid therapy, central venous catheter, contraceptives/hormone substitution, smoke; nevertheless, these risk factors do not explain the TE risk increase completely^[87].

It is well-known that in IBD patients there is not a completely explained imbalance between coagulation and fibrinolysis in favor of coagulation.

Active intestinal inflammation is not probably the unique risk determinant since about 30-40%^[87,89] of thrombosis occurs during quiescence of the IBD and proctocolectomy has not shown a very clear protective effect on recurrent venous thrombosis^[90].

Also other factors have been claimed; hyperhomocysteinemia, a well-known risk factor for venous and arterial thrombosis, occurs more often in IBD patients than in the general population and seems to be directly dependent by folate and vitamin B12 deficiency even if there is not a complete concordance in literature^[91-93].

The role of the inherited thrombophilia has been recently reviewed^[94]. The analysis has shown that the most frequent prothrombotic genetic mutations (factor V Leiden mutation, G20210A mutation in the gene of prothrombin, homozygous in the gene of methylenetetrahydrofolate reductase) are not significantly associated with IBD.

At the same time with the limit of a small number of subjects participating in the studies, it seems that there is no difference in the prevalence of genetic mutations in IBD patients with thrombosis compared with non-IBD subjects with thrombosis. Anyway a recent study comparing IBD patients with thrombosis and IBD controls has reevaluated the role of genetic factors finding a significant higher prevalence of factor V Leiden in the thrombosis group (20% *vs* 0%)^[95].

The data regarding the prevalence of antiphospholipid antibodies in IBD are conflicting, but seem to suggest an increase frequency in IBD. In limited series, the level of lipoprotein (A) has been found to be higher than in controls^[87]. No evidence exists about treating thrombotic events differently than in non-IBD patients^[87].

Conclusively many factors are suspected to play a role in the thromboembolic increased risk of IBD patients but further studies are necessary to identify their specific contribution.

At the moment in IBD patients the elimination of removable risk factors is recommendable; in case of thrombosis probably is useful to evaluate the thrombotic risk performing coagulation laboratory parameters and genetic tests.

Anemia

Anemia is a frequent extraintestinal manifestation in IBD;

about one-third of IBD patients have hemoglobin levels below 12 g/dL^[96]. The anemic state correlates strictly with the quality of life and so is certainly an important problem in the therapeutic management of chronic patients^[97]. Multiple pathogenic mechanisms often coexist in anemic patients leading to mixed features anemia. Chronic intestinal bleeding with iron loss (due to bowel inflammation) causes a hypochromic and microcytic anemia with associated hypoferremia and hypoferritinemia; the chronic inflammatory disease (typically characterized by hyperferritinemia) can cause anemia through the proinflammatory cytokine-dependent diversion of iron traffic to reticuloendothelial system and erythroid progenitor cell development interference^[98]. The same inflammatory cytokines are able to inhibit erythropoietin production^[99]. Recently, *in vitro* anti-TNF- α factors showed positive effects in preventing apoptosis of erythroid cells^[100]. Other mechanisms implied in anemia are iron malabsorption (in duodenum or upper jejunum disease CD), vitamin B12 malabsorption (in terminal ileum and gastric CD), and folate deficiency (malabsorption, inadequate diet, and side effects of sulfasalazine and methotrexate). Vitamin B12 and folate deficient anemia is characteristically macrocytic. Myelosuppressive direct effects have been reported frequently for azathioprine/6-mercaptopurine and sometimes also for sulfasalazine and 5-aminosalicylic acid^[101]. Correction of the anemic state is useful also in low-grade anemia. It is important to prevent and treat intestinal flares that are often the cause of anemia and to reintegrate the lacking of iron, B12, and folate.

In low-ferritin patients, prevention therapy with oral iron can be sufficient; in overt anemia iron intravenous (preferentially iron sucrose) supplementation should be preferred to oral route because of major efficacy and no collateral intestinal side effect. Epo therapy is useful in patients with no satisfactory response to iron therapy alone^[101]. Low levels of Epo, soluble transferring receptors or transferrin have shown to predict iron sucrose resistance^[102].

Urinary system manifestations

IBD is a risk factor for renal immune and non-immune mediated diseases.

The prevalence of nephrolithiasis in IBD varies from 2% to 6% and is more frequent in CD than in UC^[103]. Calcium-oxalate stones are the most common and are caused by hyperoxaluria due to increased intestinal absorption of oxalate. In fact in the bowel that does not absorb fatty acids link calcium preventing calcium-oxalate precipitation with the consequent increased absorbable oxalate fraction. More than one lithogenic factors are often present in the same patient, more frequently during active disease. The main lithogenic risk factors are: low urinary volume, low urinary PH, increased excretion of lithogenic substances as oxalate, phosphate, uric acid, and decreased concentration of anti-lithogenic substances as citrate and magnesium. Colectomy in UC and ileo-colonic resection in CD seems to further increase the risk of lithiasis and oxalate stone formation occurs mainly in ileal CD^[104].

Table 4 Drugs and their possible adverse side effects

Drugs	Possible adverse side effects
Corticosteroids	Acne, fluid retention, fat redistribution, hypertension, hyperglycemia, psycho-neurological disturbances, cataracts, growth failure in children, osteonecrosis ^[115]
Mesalazine	Nausea, dyspepsia, rare nephritis, rare idiosyncratic worsening of IBD ^[116]
Sulfapyridine	Headache, nausea, anorexia, rare hypersensitivity hepatitis, hemolytic anemia, pancreatitis, reversible sperm abnormalities, worsening of IBD ^[116]
Azathioprine/mercaptopurine	Pancreatitis, bone marrow suppression, hepatotoxicity ^[115]
Methotrexate	Nausea, leucopenia, hepatic fibrosis, hypersensitivity pneumonia ^[117]
Cyclosporin	Nephrotoxicity, hypertension, headache, gingival hyperplasia, paresthesias ^[115]
Tacrolimus	Nephrotoxicity ^[115]
Infliximab	Infusion reactions, delayed hypersensitivity-like reactions, drug-induced lupus, tuberculosis reactivation ^[118]
Metronidazole	Nausea, metallic taste, peripheral neuropathy ^[115]

Periodic sonographic examination is recommended for early diagnosis and for the prevention of complications. Furthermore, minimal signs of tubular damage have been found in about 20% of IBD patients but rarely they are clinically relevant^[105].

A calculus urethral obstruction, prevalently localized on the right, is also possible and is related to the mechanisms of adherence and compression by inflamed bowel (prevalently terminal ileum)^[103]. Urinary tract fistulas occur in about 1.7-7.7% of patients. They can cause pneumaturia, dysuria, recurrent infections, and fecaluria. At the moment in most cases, the non-satisfactory response to medical therapy makes surgery the best option^[106].

Clinical relevant renal amyloidosis has been reported in about 1% of IBD patients (more frequently in ileal CD). It is probably related to acute phase reaction proteins^[107]. In IBD patients, cases of glomerulonephritis causing nephrotic syndrome and renal failure have also been reported. They are related to intestinal disease activity, are quite responsive to IBD therapy and can present many patterns at histology^[108]. Moreover, it seems that minimal, clinically non significant, glomerular inflammatory changes are quite frequent in IBD patients as shown in a post mortem study (70% of subtle renal lesions *vs* 8% of controls)^[109] but following data do not exploit this aspect.

Other rare extraintestinal manifestations

It has been reported in literature about the occurrence of other rare extraintestinal manifestations of IBD, such as, chronic recurrent multifocal osteomyelitis (CRMO), myositis^[110], polyneuropathy, Guillain-Barre syndrome^[111], lymphocytic encephalomyeloneuritis^[112], myocarditis^[113], and pleuropericarditis^[114].

Drug-induced side effects

Many drugs used in IBD treatment can cause side effects involving various organs (Table 4). These effects that often need drug interruption enter in differential diagnosis with extraintestinal manifestations/complications of IBD; their early diagnosis is facilitated by periodical serum analysis exploring liver, pancreatic, renal, and hematological system integrity as for example is recommended in methotrexate and azathioprine use^[115]. Furthermore, the limited use to short period of other drugs can prevent their effect as for example as it often happens for corticosteroids (Table 4).

CONCLUSION

IBD is a systemic disease, since its clinical manifestations can affect not only the bowel but also practically any other organ (eyes, liver, osteoarticular system, kidneys, and so on) through different (often not completely cleared) mechanisms. At the moment, awareness of the high incidence of extraintestinal manifestations is often inadequate. Therefore, prevention, early diagnosis, and adequate treatment of these pathological conditions, sometimes more dramatic than the intestinal disease, are necessary to increase patients' health. Clinical interventional trials in IBD patients should consider these conditions with more attention to indicate the best cost-effective method for clinicians.

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Radiation therapy for portal venous invasion by hepatocellular carcinoma

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Abstract

AIM: To clarify the efficacy and safety of three-dimensional conformal radiotherapy (3-D CRT) for this disease and to specify patient subgroups suitable for this treatment.

METHODS: Fifty-two patients with HCC received PVI-targeted radiation therapy from January 1995 through December 2003. Portal venous invasion (PVI) was found in the second or lower order branches of the portal vein in 6 patients, in the first branch in 24 patients and in the main trunk in 22 patients. Child classifications of liver function before radiation therapy were A, B, and C for 19, 24 and 2 patients, respectively. All patients received three-dimensional conformal radiotherapy with a total dose ranging from 39 to 60 Gy (57.0 Gy in average).

RESULTS: Overall survival rates at 1, 2, 3, 4, and 5 years were 45.1%, 25.3%, 15.2%, 10.1%, and 5.1%, respectively. Univariate analysis revealed that Child status, the number of tumor foci, tumor type, transcatheter arterial embolization (TAE) after radiation therapy were statistically significant prognostic factors. Multivariate analysis showed that the number of tumor foci and TAE after radiation therapy were statistically significant.

CONCLUSION: The results of this study strongly suggest the efficacy of 3-D CRT as treatment for PVI in HCC. 3-D CRT is recommended in combination with post-radiation TAE for PVI of HCC with 5 tumor foci or less in the liver and with Child A liver function.

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Key words: Hepatocellular carcinoma; Portal venous

invasion; Radiation therapy

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INTRODUCTION

Patients with primary hepatocellular carcinoma (HCC) often develop portal venous invasion (PVI)^[1-6]. PVI is associated with a high probability of extensive tumor spread and an elevation of portal vein pressure, which subsequently may cause esophageal varices and liver dysfunction. Transcatheter arterial embolization (TAE) which is performed frequently for advanced HCC is not indicated when portal blood flow decreases due to PVI. It is, therefore, associated with a poor prognosis^[2-3]. No treatment strategy for PVI has been established, and the median survival has been reported to range only from 5 to 11 mo^[7-12]. Notably, the 1-year survival rate is less than 50% and there are only a few 3-year survivors of PVI affecting the first branch and/or the main trunk of the portal vein^[3, 13].

Few articles on the radiation therapy have been reported for the disease. The aim of the study is to clarify the efficacy and safety of three-dimensional conformal radiotherapy (3-D CRT) for PVI from HCC and to specify patient subgroups who are best benefited by this treatment strategy.

MATERIALS AND METHODS

Fifty-two patients with HCC received PVI-targeted radiation therapy from January 1995 through December 2003. The aim of the treatment was to prevent and/or improve liver dysfunction caused by PVI and re-actualize transcatheter arterial embolization (TAE) for intrahepatic tumors as well as to control PVI itself. All patients but one were male. A diagnosis of HCC was made using ultrasonography (US), computed tomography (CT), angiography, and liver biopsy, as previously reported. Histopathological diagnosis was confirmed in all the patients. Criteria of diagnostic imaging of PVT is a low-attenuation intraluminal mass that expanded the portal vein on enhanced CT or conventional US^[14-16] and/or the detection of pulsatile flow in portal vein thrombi by Doppler US^[17].

Table 1 List of size of intrahepatic tumor

Size of intrahepatic tumor (cm)	No. of patients
No HCC	1
1-1.99	2
2-2.99	21
3-3.99	11
4-4.99	7
5-5.99	3
6-6.99	2
≥7	5

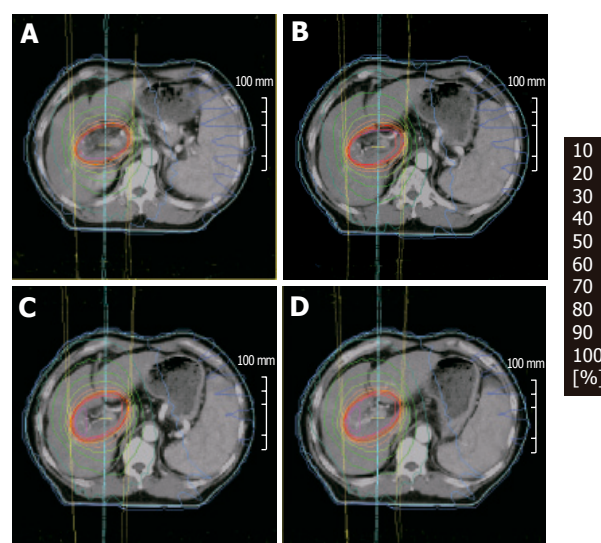
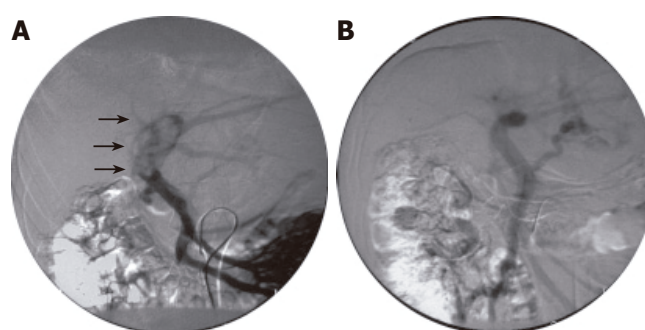
The observation period ranged between 17.4 mo and 123.6 mo (average, 60.4 mo; median, 58.4 mo). These patients ranged in age from 43 years to 82 years (average, 63.1 years; median, 64.0 years) when radiation therapy was started. Eight patients were hepatitis B virus (HBV) related, 40 patients were hepatitis C virus (HCV) related, and 45 had pathologically proven liver cirrhosis. Child classifications of liver function before radiation therapy were A, B, and C for 19, 24 and 2 patients, respectively. Values of the Karnofsky index ranged from 70 to 100 (average, 90.8; median 90). PVI was found in the second or lower order branches of the portal vein in 6 patients, in the first branch in 24 patients and in the main trunk in 22 patients. HCCs were classified as nodular, massive, and diffuse, according to the criteria of the Liver Cancer Study Group of Japan^[18]. Thirty-six patients had nodular, 9 had massive, and 6 had diffuse HCCs. The size of the intrahepatic tumors varied patient to patient and no intrahepatic tumor was detected in one patient as summarized in Table 1. Thirteen patients had a single focus for the intrahepatic tumor, 7 had 2 to 5 foci, and 31 patients had 6 or more.

Regarding treatment for HCC before the detection of PVI, 31 patients had undergone percutaneous tumor ablation (PTA) such as percutaneous ethanol injection (PEIT) and percutaneous microwave coagulation therapy (PMCT), 40 patients had undergone TAE, and 22 patients had both procedures. Tumors were surgically resected in 7 patients.

All patients received 3-D CRT with a multi-leaf collimator (MLC) using 6 MV X-ray. Figure 1 is an example of dose distributions in one patient. Our goal to give a total dose of 60 Gy to each patient was achieved in 38 cases (mean total dose, 57.0 Gy, range: 39-60 Gy). A daily dose of 2 Gy was delivered 5 times a wk. Clinical tumor volume (CTV) was the tumor itself which was depicted on CT and/or US. Planning target volume (PTV) was defined as 1.5 to 2.0 cm beyond the enhanced lesion on CT scan. Radiation was performed while the patient was breathing shallowly without the use of breath control or respiratory gating. Initial effects of radiation therapy on PVI was evaluated 8 to 12 wk after the completion of radiation and expressed according to the WHO criteria^[19].

After the completion of radiation therapy for PVI, 10 underwent PTA, 25 underwent TAE, and 8 patients had both procedures. No surgical operation was performed following radiotherapy for PVI.

The survival period was measured from first day of irradiation and the Kaplan-Meier technique was used to

**Figure 1** An example of dose distributions (A, B, C, D) in 3D-CRT.**Figure 2** Angiographs of the portal vein showing disappearance of a tumoral embolus after irradiation therapy. Angiographs taken before and after treatment are shown to the left (A) and right (B), respectively.

calculate the survival rate. The differences in survival rates were analyzed using the log rank test. Uni and multivariate analyses were performed by Cox's proportional hazard model. Statistical analyses were considered significant if the *P*-value was 0.05 or less.

Written informed consent was obtained in all the patients in this study.

RESULTS

The initial effects of radiation therapy on PVI were complete regression (CR) in 2 patients, partial regression (PR) in 24 patients, stable disease (SD) in 18 patients and progressive disease (PD) in 8 patients. Figure 2 shows angiographs of the portal vein before (A) and after irradiation (B). Defect of the portal blood flow which was marked with arrows (A) disappeared after the treatment (B). Intrahepatic tumors increased in 31 patients (59.6%).

Overall survival rates at 1, 2, 3, 4, and 5 years were 45.1%, 25.3%, 15.2%, 10.1%, and 5.1%, respectively (Figure 3). Neither age, nor Karnofsky performance index was related to the survival rate. Survival time greatly depended on

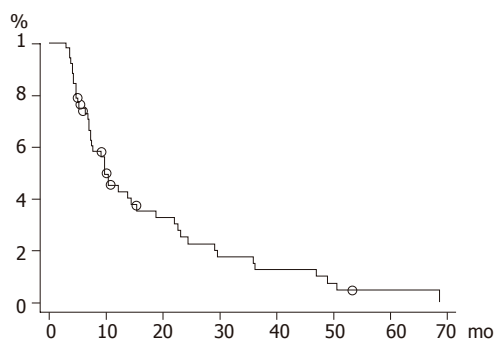


Figure 3 Overall survival curve.

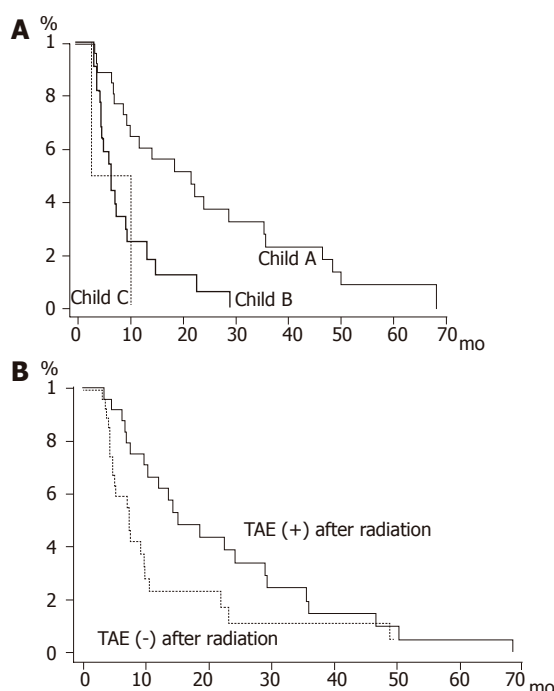


Figure 4 **A**: Survival curves according to Child classification. Solid line: Child A; bold line: Child B; dashed line: Child C; **B**: Survival curves for the patient group with and without post-radiation TAE. Solid line: post-radiation TAE (+); dashed line: post-radiation TAE (-).

the Child status as shown in Figure 4A ($P = 0.0007$). Diffuse type HCC showed a significant lower survival rate compared with nodular or massive type HCC ($P = 0.0389$). We also noted a higher survival rate in cases with 5 foci or less in the liver ($P = 0.01$). The spread of PVI (invasion of the main trunk, first branch, and second or lower order branches) was not relevant to the survival rate at all ($P = 0.7679$) (Figure 4B).

Treatment preceding irradiation, namely, surgical resection, PTA and TAE, was irrelevant to the prognosis as well. We noted a significant longer survival periods in cases with favorable primary effects following radiation therapy ($P = 0.0419$). Regarding the effect of treatments given after radiation therapy, PTA had no influence on the survival rate ($P = 0.3987$). On the other hand, the survival rate was higher, *albeit* marginally, in the TAE-treated group

Table 2 Univariate analysis

	Hazard ratio	95%CI	P value
Child classification (A)	0.217	0.048-0.978	0.0015
No. of HCC (≤ 5)	0.411	0.204-0.828	0.0129
Tumor type (diffuse)	2.897	1.147-7.313	0.0244
No TAE after RT	2.406	1.001-5.786	0.0498
Initial effect (CR)	0.194	0.043-0.878	0.0625
No surgery before RT	0.6	0.247-1.456	0.2589
No PTA before RT	1.373	0.741-2.544	0.3136
No TAE before RT	0.718	0.358-1.438	0.3494
No PTA after RT	1.372	0.654-2.880	0.4026
KPS (< 90)	1.252	0.483-0.262	0.6457
age	1.008	0.971-1.046	0.681
Main trunk invasion	1.049	0.573-1.920	0.8772

CR: complete regression; PR: partial regression; SD: stable disease; PD: progressive disease; TAE: transcatheter arterial embolization; RT: radiation therapy; HCC: hepatocellular carcinoma; PTA: percutaneous tumor ablation; CI: confidence interval.

Table 3 Multivariate analysis

	Hazard ratio	95%CI	P value
No. of HCC (≤ 5)	0.414	0.188-0.912	0.0285
No TAE after RT	3.206	1.463-7.024	0.0364
Child classification A	0.729	0.111-4.667	0.0864
Initial effect (CR)	0.314	0.059-1.664	0.5783
Tumor type (diffuse)	1.309	0.190-9.011	0.7019

CR: complete regression; PR: partial regression; SD: stable disease; PD: progressive disease; TAE: transcatheter arterial embolization; RT: radiation therapy; HCC: hepatocellular carcinoma; CI: confidence interval.

than in the TAE-untreated group ($P = 0.0465$).

Univariate analysis by the Cox proportional hazard model revealed that Child status, the number of tumor foci, tumor type, TAE after radiation therapy were statistically significant prognostic factors (Table 2). Multivariate analysis by Cox's proportional hazard model using five variables, namely, Child status, the number of tumor foci, tumor type, TAE after radiation therapy, initial effect showed that the number of tumor foci in the liver ($P = 0.0285$) were the most important factors contributing to the eligible prognosis of patients with HCC invading the portal vein, followed by TAE after radiation therapy ($P = 0.0364$) (Table 3).

Hepatic function, expressed using the Child classification, remained unaltered in 34 cases after radiation therapy. In the remaining 18 patients the classification changed from Child A to B in 13 cases, from Child B to C in two cases, and from Child B to A in three cases. A patient whose liver function deteriorated from Child B to C lapsed into a hepatic coma after radiation therapy. No other serious side effects of irradiation were observed.

DISCUSSION

PVI has been considered to be an important contributor to a poor prognosis in primary HCC, and no definitive treatment strategy has been established to it. The results of the present study demonstrated a relatively favorable survival following radiation therapy for PVI. The 3-year survival rate of 15.2 % among the present study patients

is excellent in comparison with survival rates reported previously^[8-9]. In contrast to the surgical series^[3,13], in the present study, 3-year survival did not depend on whether PVI invaded the first branch and/or the main trunk of the portal vein. The authors consider, therefore, that radiation therapy seems to be superior to surgical intervention for the patients who developed PVI in the proximal portal vein.

Survival rates did not differ significantly among groups of patients who received one of three different treatments prior to radiation therapy, namely surgical resection, PTA and TAE. On the other hand, a significantly favorable outcome of TAE after radiation therapy was found. The TAE was performed preferentially in patients whose PVI was controlled sufficiently and whose liver function was kept relatively normal; thus, this bias in selecting patients for TAE was probably the main reason for the favorable outcome. However, 6 of 7 patients who survived for more than 2 years had undergone TAE after radiation therapy. Therefore, it appears beneficial to perform TAE in cases in which PVI is under sufficient control.

Radiation therapy for hepatic carcinoma is not performed widely, and reports of such treatment are infrequent^[20-23]. Reports of radiotherapy for PVI from HCC is even rare^[24-26]. Radiation therapy markedly damages normal hepatic cells when used with conventional large fields. According to the experience in the present paper, however, decreases in liver function were within the acceptable range if only PVIs of the patients classified into Child A and B were treated with 3-D CRT. With 3-D CRT, high doses of radiation are confined to the target region while surrounding areas of the liver are exposed only to lower doses. Although clinical data have accumulated concerning hepatic dysfunction after whole or partial irradiation of the liver, those data are not directly applicable to predict hepatic dysfunction after conformal radiotherapy. The biological effect on the liver of such inhomogenous irradiation has not been elucidated fully. Further accumulation of clinical experience is required to determine the most appropriate method of irradiation for this disease.

The results of this study strongly suggest the efficacy of 3-D CRT as treatment for PVI in HCC. From the present analyses, the authors will recommend 3-D CRT in combination with post-radiation TAE for HCCs with 5 tumor foci or less in the liver and with Child A liver function.

Needless to say, because this study was retrospective, the presence of bias in the selection of treatments for individual patients cannot be excluded completely. To more fully clarify the significance of this therapy, a prospective randomized trial should be performed. We hope that the outcomes reported here may contribute to designing future clinical trials and optimizing treatment for this disease.

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Effect of oral *Lactococcus lactis* containing endostatin on 1, 2-dimethylhydrazine-induced colon tumor in rats

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Abstract

AIM: To investigate the effects of oral *Lactococcus lactis* (*L. lactis*) containing endostatin on 1, 2-dimethylhydrazine (DMH)-induced rat colorectal cancer.

METHODS: Recombinant endostatin was produced by the expression of *L. lactis* NZ9000. Sixty male Wistar rats were injected with DMH (40 mg/kg body weight) subcutaneously once a week for 10 wk to induce colorectal cancer. The rats were gavaged with 1 mL of endostatin at a dose of 1×10^8 /d and fed with the basal diet. The animals were killed after 22 wk for histopathological examination. The total time of experimental observation was 58 wk.

RESULTS: Rat endostatin protein was expressed in *L. lactis*. Recombinant endostatin exhibited a significant effect on colorectal cancer ($P < 0.05$). Furthermore, the mean survival time of the rats treated with endostatin was longer than that of the animals treated with DMH. There was no statistically significant difference between the rats treated with endostatin and those treated with DMH. The results showed that endostatin could not result in complete cure.

CONCLUSION: Oral endostatin exerts an influence on the progression of chemically induced colon tumors.

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Key words: Endostatin; DMH; Tumors

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INTRODUCTION

There are lines of evidence that angiogenesis is essential for the growth and persistence of solid tumors and their metastases^[1,2]. Tumor angiogenesis is regulated by the balance between proangiogenesis and antiangiogenesis factors, and this balance varies in different organ environments^[3]. Systemic administration of recombinant endostatin potently inhibits angiogenesis and maintains metastases at a microscopic size, resulting in a strong anti-tumor activity^[4-6]. Endostatin has been shown in some studies to inhibit the formation or growth of lung and liver metastases^[7,8]. Endostatin, an angiogenesis inhibitor produced by hemangioendothelioma, is a 20 kDa carboxy-terminal fragment of collagen XVIII^[5]. The efficacy of endostatin in colon environment is not well established. To our knowledge, there are no published reports on the efficacy of endostatin against chemically induced colon tumor progression.

An autochthonous colon cancer model is useful to evaluate the clinical therapeutic efficacy of drugs for colorectal cancer^[9,10]. As DMH model is known to closely parallel the human disease in terms of disease presentation, gross and microscopic pathology^[11], it is anticipated that DMH-induced colon tumors respond to chemotherapeutic drugs. Drugs such as 5-fluorouracil (5-FU) can inhibit the growth of DMH-induced colon tumors and prolong the survival of their rodent hosts^[12]. Therefore, DMH-induced colon tumors at present are the most popular models to study the morphology, pathogenesis, prevention, and treatment of colorectal cancer^[11,13]. Though 5-FU derivatives have been tested in the DMH model, whether this colon cancer model of rats can be applied to the evaluation of the effect of endostatin on colon tumors remains unknown. The aim of the present study was to investigate the effects of recombinant endostatin on the progression of DMH-induced colon tumors in rats.

MATERIALS AND METHODS

Animals and chemicals

Five-week-old male Wistar rats were provided by the Laboratory Animal Center, Chinese Academy of Medical Sciences, Beijing, China, and housed in plastic cages in a 12-h light/dark cycle at 22 ± 2 °C and $44 \pm 5\%$ relative humidity. Rats were fed with the basal diet with free access to water. Body weight and food consumption were measured weekly during the experiments. DMH was purchased from Tokyo Kasei Co. (Tokyo, Japan).

Preparation of recombinant endostatin

Endostatin expression experiments were performed with *L. lactis* NZ9000 (donated by Institute National de la Recherche Agromique, France). All cloning steps were done with *E. coli* Top 10. *E. coli* (stored in our laboratory, China) was grown on Luria-Bertani (LB) medium and incubated at 37 °C. *L. lactis* was grown on M17 medium containing 0.5% (wt/vol) glucose and incubated at 30 °C. When appropriate, chloramphenicol was added at a final concentration of 10 µg/mL and ampicillin was supplied at a concentration of 100 µg/mL. Expression of the endostatin gene was induced by nisin promotor. *L. lactis* was cultured overnight and then transferred into a fresh medium at a dilution of 1:50. After 3–4 h of incubation, 1 µg/mL of nisin (Sigma) was added to the culture and incubated for 3–4 h.

Cloning and expression of rat endostatin in *L. lactis*

Total RNA was extracted from rat kidney tissue using the SV total RNA isolation systems kit (Promega) and reverse-transcribed by reverse transcription system kit (Promega). The mixture was incubated at 25 °C for 10 min, at 42 °C for 60 min, at 95 °C for 5 min, and at 4 °C for 5 min. The two gene-specific primers were designed by the sequence encoding the carboxy terminal portion of rat collagen XVIII. The primer sequences were 5'-TTT GAA TTC GCC CAC ACC CAC CGC GAC TTC CAG CCG-3' and 5'-AAA AGC GGC CGC CTA CTT GGA GGC GGC AGT CAT GAA GCT-3'. PCR was performed in a total volume of 50 µL of reaction solution and 2 µL of RT template. The PCR conditions were as follows: denaturation at 94 °C for 5 min, then 25–35 cycles at 94 °C for 0.5 min, at 56 °C for 0.5 min, at 72 °C for 1 min, a final extension at 72 °C for 5 min and a DNA fragment was obtained. The amplified fragment was purified using the QIAquick PCR purification kit (QIAGEN Inc.) and digested with *EcoRI* and *NotI*. The resulting fragment was respectively ligated to nisin promotor plasmids pLA141 and pLA151 digested with *EcoRI* and *NotI*. The pLA141 plasmid carried a signal peptide Usp45. The recombinant plasmid DNA was transferred into *L. lactis* NZ9000 by electroporation and transformants were plated on GM17 agar plates containing chloramphenicol according to the method of Wells *et al.*^[15]. The recombinant plasmid DNA was isolated from *L. lactis* as described previously^[15,16] and the nucleotide sequence was further determined.

Western blotting

Total protein was prepared from exponentially growing cultures. The bacteria were harvested by centrifugation at 3 000 r/min for 10 min at 4 °C, washed with PBS, resuspended in 1 mL of 10 mmol/L Tris-HCl (pH 7.5) and disrupted with a French press (Bioritech). The cell suspension was centrifuged at 10 000 g for 10 min at 4 °C to remove cell debris. The samples were mixed in *Laemmli* buffer and subjected to SDS-12% polyacrylamide gel electrophoresis. The protein was transferred onto nitrocellulose membranes with a Bio-Rad electroblotter. The blots were developed with BCIP/NBT developing

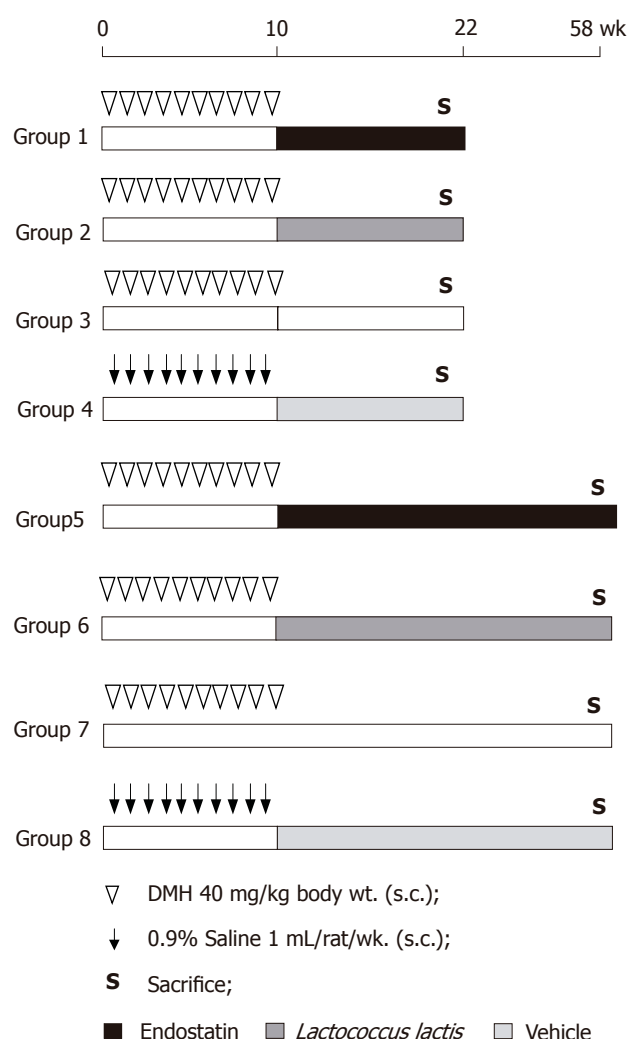


Figure 1 Experimental design.

buffer (Sigma).

Treatment protocol

The experimental design is shown in Figure 1. After 1 wk of acclimatization, 80 six-week-old rats were randomly divided into 8 groups (10 rats/group). Animals in groups 1–3 and 5–7 received subcutaneous injections of DMH dissolved in normal saline solution (40 mg/kg body weight) once a week for 10 wk. Rats in groups 4 and 8 were injected with 0.9% normal saline (vehicle) at the same time. After the last DMH treatment, the animals were additionally gavaged with 1 mL of *L. lactis*-secreted endostatin protein (groups 1 and 5) and *L. lactis* without endostatin gene but containing plasmids (groups 2 and 6) once a day for 12 wk or until they were killed after 58 wk. The dose of endostatin and *L. lactis* was 1×10^8 daily. Groups 4 and 8 were gavaged with 1 mL of the solutions not containing *L. lactis* (the vehicle control). Groups 3 and 7 served as a carcinogen control. The time of treatment differed slightly in each experiment. All animals that survived were killed under either anesthesia at wk 22 (groups 1–4) or to end of the experiment. The total time of experimental observation was 58 wk.

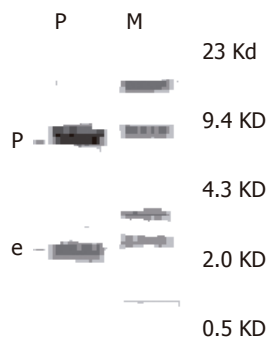


Figure 2 Expression plasmid obtained by restriction enzyme analysis. P: expression plasmid cut by *EcoRI* and *Not I*; e: endostatin gene; M: molecular weight marker.

Autopsy

The colons were removed, flushed with saline and opened along the longitudinal median axis. Tumor width (*W*) and length (*L*) were measured with calipers. The tumor volume (TV) was determined by the following formula: $TV = (L \times W^2) / 2^{[8]}$. After the gross pathologic changes (number, dimensions, and distribution of the tumors) were recorded, the colons were fixed flat in 10% phosphate-buffered formalin. The liver and kidneys were removed and weighed. Other major organs (stomach, small intestine, spleen, lungs, and lymph nodes) were also excised and fixed in 10% phosphate-buffered formalin. All tissues were embedded in paraffin, cut into sections and stained with hematoxylin and eosin. The proximal, intermediate and distal segments of the colon were examined for histopathological analysis.

Tumor staging

Animals with DMH-induced colon cancer developed multiple tumors and each tumor had a different histological stage^[14]. Consequently, the animals were staged (Duke's stage) with reference to a single index tumor, defined as the largest macroscopically and histologically identifiable colon tumor.

Statistical analysis

Statistical analyses were completed with SPSS 9.0 software. The significance of differences between the average values of the groups was analyzed using Cochran's two-tailed Student's *t*-test. The significance of differences in lesion incidence between the groups was assessed by χ^2 test. Rat mortality was analyzed by the log-rank method of Peto *et al.*^[17].

RESULTS

Construction of expression plasmid

The plasmid containing the endostatin gene was pla148, identified as the expression plasmid I. Recombinant *L. lactis* was obtained by PCR and restriction enzyme analysis when the recombinant plasmid was transformed into *L. lactis* by electroporation (Figure 2).

Expression of rat endostatin gene in *L. lactis*

Recombinant lactic acid bacteria were incubated and

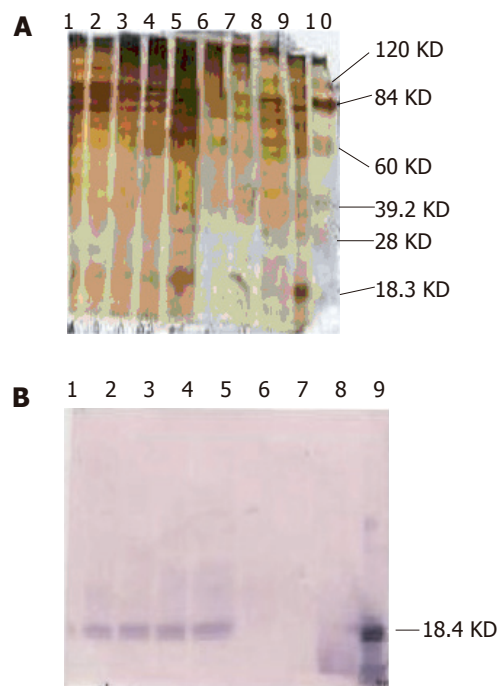


Figure 3 Expression of rat endostatin gene in *L. lactis*. **A:** Silver-stained SDS-PAGE of expressed endostatin in *L. lactis*; **B:** Western blot. Lanes 1-6: engineered *L. lactis* 1-6 h after induction; lane 7: *L. lactis* without endostatin gene; lane 8: rat sera from groups after oral recombinant *L. lactis*; lane 9: endostatin protein expression in *E. coli* cells.

induced by *nisin* in M17-Glu for 6 h. Endostatin protein was identified by SDS-PAGE and Western blot with the antibody prepared from rabbits immunized with human endostatin protein. Rat endostatin protein was expressed in *L. lactis* (Figures 3A and 3B).

Animal experiment

All rats in groups 1-4 and 8 survived until the final termination and were relatively healthy throughout the experiment. No signs of severe toxicity were observed in all the animals that were given endostatin. No tumor was found in vehicle-treated animals. By the end of wk 22, the average body weights of the rats treated with DMH or endostatin or *L. lactis* were significantly decreased compared to the vehicle control ($P < 0.05$). Relative liver and kidney weights and food consumption did not significantly differ among the groups (Table 1). Macroscopically, the distribution of colon tumors in the proximal and middle colon at the end of wk 22 had no significant difference among the groups (data not shown).

Histopathological findings are summarized in Table 2. Colon epithelial lesions were divided into adenomas and carcinomas. At the end of wk 22, the incidence of colon tumors was not significantly affected by endostatin. The mean tumor incidence in a single tumor-bearing rat was 2.50 in group 1 and 4.00 in group 3. Tumor volume was decreased in rats receiving endostatin. However, it did not differ from that in DMH-treated group. In addition, there was a significant difference in Duke's stage between the animals treated with DMH and those treated with

Table 1 Average final body weight, relative liver and kidney weights, and food consumption data (mean±SD)

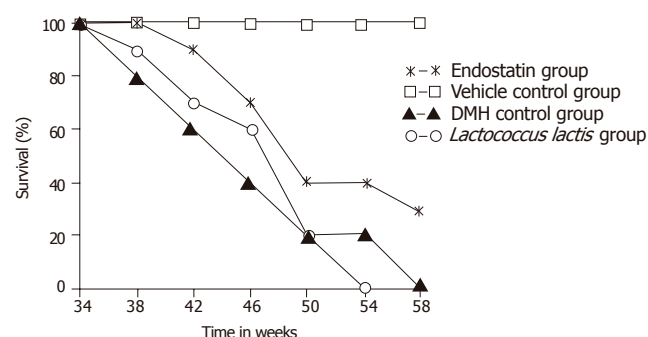
Group	Treatment	<i>n</i>	Final body Weight, g	Relative liver Weight, g	Relative kidney Weight, g	Food consumption (g/rat/d)
1	DMH+endostatin	10	379.0±24.9 ^a	2.94±0.26	0.56±0.12	18.02
2	DMH+ <i>L. lactis</i>	10	395.0±36.5 ^a	3.05±0.25	0.56±0.08	18.00
3	DMH	10	383.5±19.2 ^a	3.10±0.40	0.55±0.07	18.48
4	Saline+vehicle	10	439.5±39.3	3.09±0.35	0.56±0.12	20.07

^a*P*<0.05 vs group 4.**Table 2** Colon tumor incidence, classification, multiplicity, tumor volume, and stage in rats treated with DMH with or without endostatin (mean±SD)

Treatment	<i>n</i>	Incidence	Adenoma	Carcinoma	Multiplicity number	Tumor volume mm ³	Duke's stage		
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)			A	B	C
DMH+endostatin	10	10(100)	5 (50)	5 (50)	2.50±1.80	2.35±1.84	1	4	-a
DMH+ <i>L. lactis</i>	10	9(90)	2 (22)	7 (78)	2.67±1.47	2.54±2.00	4	6	-a
DMH	10	10 (100)	5 (50)	5 (50)	4.00±2.96	4.31±4.56	2	-	3

^a*P*<0.05 vs DMH-treated rats.**Table 3** Tumor classification, distribution, and differentiation in rats treated with DMH with or without endostatin

Treatment	Tumor number	Classification (%)		Distribution in colon (%)			Differentiated carcinoma (%)		
		Adenoma	Carcinoma	Proximal	Middle	Distal	Well	Moderately	Poorly
DMH+endostatin	25	16 (64)	9 (36)	12 (48)	9 (36)	4 (16)	6 (66.7)	3 (33.3) ^a	0
DMH+ <i>L. lactis</i>	24	15 (62.5)	9 (37.5)	10 (41.7)	12 (50)	2 (8.3)	6 (66.7)	3 (33.3) ^a	0
DMH	40	27 (67.5)	13 (32.5)	15 (37.5)	19 (47.5)	6 (15)	3 (23.1)	10 (76.9)	0

^a*P*<0.05 vs DMH-treated rats.**Figure 4** Survival rate of rats injected with DMH with or without endostatin and normal saline.

endostatin (*P*<0.05). Liver lesions and lymph node metastases were observed in about 30% of the animals in group 3.

The survival rates of rats in groups 5-8 are shown in Figure 4. The group that received endostatin had a survival rate of 30%. The survived rats were killed and metastases were found in their lungs and livers. All the saline-injected rats were alive at the end of the experiment. However, none of the DMH-treated rats survived the full duration of the experiment. The mean survival time of endostatin-treated animals was longer than that of DMH-treated rats. The range of ages at death in DMH-treated animals was 38-57 wk (Table 3).

DISCUSSION

Studies using preclinical models of nonhematologic malignancies indicate that antiangiogenic therapies may delay or even abrogate tumor growth^[5,18,19]. Endostatin is one of the antiangiogenic drugs and our data indicate that the administration of endostatin after DMH treatment could prolong the survival time of rats. The Duke's staging system for human colorectal cancer provides accurate prognostic information. In other words, animals with less advanced disease (stage A) survive significantly longer than those with advanced disease (stages B and C) irrespective of the treatment. In our study, there was a significant difference in the levels of differentiation and metastases (Duke's stage) between the animals treated with DMH and those treated with endostatin. Endostatin-treated rats had an improved survival compared to untreated rats, indicating that the survival time of rats with colon cancer parallels to that of human beings with this disease. These results can at least in part explain the mechanism of the potent antiangiogenic and antitumor activities of endostatin. Furthermore, the improved survival is directly attributable to the effective induction of tumor stabilization and its ability to inhibit specifically endothelial proliferation in endostatin-treated animals. However, oral administration of endostatin could only prolong the survival time of tumor-bearing rats but not result in complete cure.

There are reports on endostatin against metastases in lung, stomach, and liver^[7,8,20]. Recent studies showed that endostatin has an antiangiogenic action^[21-24] and can

induce apoptosis in colon cancer cells by inhibiting tumor angiogenesis and inhibit tumor growth and metastases of human colon cancer xenograft in nude mice^[24]. Some other mechanisms may be involved in endostatin stabilizing and maturing newly formed blood vessels^[21]. Jia *et al.*^[22] reported that endostatin could inhibit tumor growth and angiogenesis by blocking Vegf/Flk-1 pathway. In addition to its antiangiogenic activity, endostatin exerts a direct anticancer action that appears to be restricted to some tumor cell lines^[25]. At the same time, endostatin has been demonstrated to induce regression of tumors in mice^[7,26], but actual regression as opposed to growth inhibition has not been demonstrated in the colon environment. Though our study demonstrated that endostatin could influence rat colon tumor progression, the precise mechanisms by which endostatin exerts effects on colon carcinoma are not well understood.

Long-term administration of endostatin is needed because the inhibition of tumor metastases has not been observed after a shorter endostatin-treated period. The ultimate goal of antiangiogenic therapy is to induce long-term tumor stabilization^[27] because data from studies in nonhuman primates indicate that endostatin may be administered for a long time without toxicity^[28]. Furthermore, studies *in vitro* and *in vivo* suggest that endostatin gene therapy can effectively suppress angiogenic processes in model systems^[29,30]. It was reported that endostatin treatment is not associated with any recognizable vascular changes in tumor samples^[31].

Certain strains of lactic acid bacteria have been found to prevent putative preneoplastic lesions induced by carcinogens^[32,33]. The antimutagenic activity of lactic acid bacteria is suspected to reside in the cell wall^[34] as lactic acid itself has no antimutagenic effect^[35]. The findings of the current study do not support the suggestion that the addition of *L. lactis* may also prolong the survival time of DMH-treated rats. The reason for this is unclear, but might be explained by the differences in bacterial strains. The antitumor effect of lactic acid bacteria is still controversial^[36]. Further study is needed to identify the antitumor effect of endostatin and the precise mechanisms by which these effects are mediated.

In conclusion, long-term administration of endostatin can inhibit the progression of chemically-induced colon tumors and prolong the survival time of rats.

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MORT1/FADD is involved in liver regeneration

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INTRODUCTION

Liver regeneration upon extensive liver damage is critical to survive diseases, such as fulminant hepatitis without liver transplantation. Beyond the cause of the underlying liver disease, it is well accepted that death receptor-mediated hepatocyte apoptosis is an important mechanism for liver damage^[1,2]. The cascade of intracellular signaling events during apoptosis is reasonably well understood. Of critical importance is the activation of caspases, the subsequent cleavage of different death substrates and finally the disintegration of the cell. In this setting, MORT1/FADD is a central adaptor molecule for a number of death receptors to recruit and activate the initiator caspase-8 upon ligand-mediated aggregation^[3,4]. Gene targeting of MORT1/FADD^[5,6] or expression of a dominant negative mutant^[7,8] confers resistance to CD95-mediated apoptosis in T cells. Recently, we have demonstrated that MORT1/FADD is also pivotal for CD95- and TNF-mediated apoptosis in hepatocytes *in vivo*^[9]. In addition, emerging evidence suggests that death receptors and their intracellular signaling proteins have a dual role and may also be involved in the regeneration and repair mechanisms. TNF receptor I (CD120a) has been found to induce proliferation in a number of cells^[10] and is involved in the initiation of liver regeneration^[11]. Even the prototypical death receptor CD95 was described to exert non-apoptotic functions^[12], e.g., stimulation of liver regeneration after partial hepatectomy (PH)^[13]. In line with this observation, mice with the *lpr* genotype and decreased expression of CD95 showed a delayed regenerative response after PH^[13]. In addition, the most upstream signaling molecules of the intracellular death pathway MORT1/FADD and caspase-8 have been described to be critical for T-cell proliferation^[5,6,14]. To our knowledge, the mechanism by which MORT1/FADD contributes to T-cell proliferation is not completely elucidated yet. The regulatory function of MORT1/FADD seems to be structurally independent of the death domain and its role in apoptosis, but to rely on phosphorylation at serine residue S191, which has been shown to be critical for the regulative role of MORT1/FADD in cell proliferation^[15].

Abstract

AIM: To explore the role of the adaptor molecule in liver regeneration after partial hepatectomy (PH).

METHODS: We used transgenic mice expressing an N-terminal truncated form of MORT1/FADD under the control of the albumin promoter. As previously shown, this transgenic protein abrogated CD95- and CD120a-mediated apoptosis in the liver. Cyclin A expression was detected using Western blotting. ELISA and RT-PCR were used to detect IL-6 and IL-6 mRNA, respectively. DNA synthesis in liver tissue was measured by BrdU staining.

RESULTS: Resection of 70% of the liver was followed by a reduced early regenerative response in the transgenic group at 36 h. Accordingly, 36 h after hepatectomy, cyclin A expression was only detectable in wild-type animals. Consequently, the onset of liver mass restoration was retarded as measured by MRI volumetry and mortality was significantly higher in the transgenic group.

CONCLUSION: Our data demonstrate for the first time an involvement of the death receptor molecule MORT1/FADD in liver regeneration, beyond its well described role as part of the intracellular death signaling pathway.

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Key words: MORT1/FADD; CD95; TNF; Apoptosis; Liver regeneration

The role of MORT1/FADD in liver regeneration has not been described so far. We used transgenic mice expressing a dominant negative mutant of MORT1/FADD in a liver-specific manner to investigate a possible role of the adaptor molecule in liver regeneration. We observed a delayed onset of liver proliferation upon PH compared to wild-type littermates and found a higher early postoperative mortality in dnMORT1/FADD transgenic mice. The increased rate of mortality might be due to initially retarded liver regeneration and underlines the critical role of MORT1/FADD in the complex network of signaling proteins which orchestrate liver regeneration.

MATERIALS AND METHODS

Animal model of liver regeneration

Heterozygous transgenic mice (strain CB6F1) expressing liver-specific dominant negative mutant of the adaptor protein MORT1/FADD under the control of the albumin promoter were generated as previously described^[9]. All animals were bred at the animal facility of the University of Mainz, had *ad libitum* access to water and food under standard conditions with 12-h dark/light cycle. All experiments were done in accordance with the Federal law and were approved by the Local Committee for Experimental Animal Research.

Partial hepatectomy

Male transgenic and wild-type animals at 6–8 wk of age were fasted overnight and operated in the morning. Mice were anaesthetized with subcutaneous injection of a solution containing 0.16% xylazine and 1.2 mg ketamin (200 µL/20 g body weight). A 70% PH was performed by ligating and removing the left and median lobe at its root. The mice were killed at different time points and livers were harvested. The livers were either shock-frozen in liquid nitrogen and stored at -80 °C or fixed at 4 °C in 40 g/L paraformaldehyde overnight for further use. Serum was obtained by heart puncture.

DNA-synthesis measurement of BrdU incorporation and mitosis

After PH, the mice were treated at different time points with 1 mmol/L BrdU (10 g/kg body weight) 3 h prior to killing. The livers were harvested and shock-frozen. The livers were cut into 5-µm-thick slides with a microtome. BrdU incorporation was measured using the *In situ* Cell Proliferation Kit (Roche Diagnostics GmbH, 82372 Penzberg, Germany) according to the manufacturer's instructions. Results were presented as an average percentage of BrdU-positive cells counted in at least two fields of each slide under 10× high power field. Mitosis of the was counted by two independent observers in a blinded fashion.

Cytokine assays

Cytokine concentrations were assessed using ELISA, OptEIA Mouse IL-6 Set (Pharmingen, BD Biosciences, 69126 Heidelberg, Germany) following manufacturer's

instructions. Samples were analyzed with an Elisa reader (MRX TC II, Dynex Technologies Lim., West Sussex BN).

Western blotting for cyclin A

Western blot analysis was performed with whole liver extract lysed in a lysis buffer. Protein concentration was equilibrated using Bradford assay reagent. The samples were boiled with sodium dodecyl sulfate sample buffer and electrophoresed on a 100 g/L sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, the samples were blotted onto a PVDF-membrane (Pall, Germany), and the membrane was blocked with 20 g/L milk powder for 30 min, followed by an overnight incubation with a rabbit anti-cyclin A serum (Anti-rabbit polyclonal Cyc A C-19, # sc596, Santa Cruz) at 4 °C with phosphate-buffered saline/Tween (0.1%) with 20 g/L milk powder (PBSTM). After washing, the membranes were incubated for 45 min with horseradish peroxidase-conjugated goat-anti-rabbit serum (dilution 1:3 000) in PBSTM. Blots were developed using the Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA, USA).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on a Magnetom Vision whole body scanner (Siemens Medical Solutions, Germany) at 1.5 T. The scanner was equipped with an experimental gradient system with maximum gradient field strength of 50 mT/m and a slew-rate of 160 mT/m/ms. A circular polarized small loop coil with 4 cm as diameter was used for imaging the mice.

For the imaging of the liver, a T1-weighted spinecho sequence with fat saturation and TR/TE/α = 640 ms/14 ms/90 °C was used. The experimental gradient system allowed a high resolution of 0.39 mm×0.39 mm with a slice thickness of 1 mm at a 50 mm×100 mm field of view. The gap between the slices was 0.8 mm.

Post processing was done using the Java-based free software ImageJ (National Institutes of Health, USA, download at <http://rsb.info.nih.gov/ij>). After drawing a region of interest (ROI) following the borders of the liver in every slice, the liver volume was calculated from the sum of pixels in each ROI over all slices with respect to the pixel size, the slice thickness and the slice gap is as follows:

$$\text{Volume}_{\text{liver}} = \sum_{\text{slice}} \sum_{\text{pixels}} \text{size}_{\text{pixel}} (\text{thickness}_{\text{slice}} + \text{gap}_{\text{slice}})$$

RT-PCR and light cycler

RNA was isolated from the cells using the First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics GmbH, 68305 Mannheim) according to the manufacturer's instructions. Primers for IL-6 were purchased from Metabion GmbH, D-82152 Planegg-Martinsried, Germany.

Duplicate PCR amplifications were carried out in a Light Cycler Fast Start DNA Master Green ITM (Roche Diagnostics GmbH, 68305 Mannheim) using Fast Start Light CyclerTM DNA Master containing Taq-polymerase, reaction buffer, and dNTPs (Roche). All reactions

were performed in 10 μ L volumes and fluorescence quantification was calculated with the aid of built-in Light Cycler software, version 3.01 (Roche).

For quantification of the mRNA, we chose the method of relative quantification with external standards (HPRT-specific primers).

Liver enzymes

Liver enzymes in serum were measured with a Roche Hitachi 917.

Hematoxylin-eosin staining

Liver samples were fixed overnight at 4 °C with 40 g/L paraformaldehyde. The tissue was then dehydrated, embedded in paraffin, and 5- μ m sections were stained with HE. Mitosis of the cells was counted by two independent observers in blinded fashion under 10 \times high power fields (about 50 cells/field).

RESULTS

A delayed proliferative response in dnMORT1/FADD mice after partial hepatectomy

As previously described, PH was performed by resection of around 70% of the total liver volume. DNA synthesis as a marker of the regenerative response was measured by BrdU staining. Kinetics of BrdU staining after PH obviously differed between transgenic and wild-type animals (Figure 1A). We observed a significantly more BrdU-positive cells in wild-type animals after 36 h, which was attenuated after 48 h (Figure 1A). In line with this observation, the number of mitoses differed in regenerating liver 48 h after PH (Figure 1B). Differences were also observed in expression levels of cyclin A at 36 h after hepatectomy (Figure 1C), when the expression was detected only in wild-type mice. These observations were further substantiated by MRI technique (Figure 1E), which allowed to follow closely the kinetic of liver mass restoration after hepatectomy. Here we could demonstrate that the delayed onset of liver regeneration was paralleled by a retarded gain of liver volume (Figure 1D). After all, surviving transgenic animals finally also reached complete volume restoration around at d 10 after PH, as did wild-type animals.

IL-6 levels after partial hepatectomy

IL-6 has been shown to be an essential factor during initial liver regeneration^[16]. Wuestefeld *et al.*^[17] specified the effect of IL-6 and demonstrated an important hepatoprotective role of IL-6 during the early phase of liver regeneration. We therefore investigated possible differences in IL-6 levels after PH. Serum IL-6 levels peaked around 24 h after PH (Figure 2A), however, did not significantly differ between wild-type and transgenic animals. Interestingly, a significantly higher IL-6 mRNA expression was detected in transgenic livers 36 h after hepatectomy (Figure 2B). This observation demonstrated a functional IL-6 deficiency to be unlikely and rather pointed to a compensatory reaction towards delayed

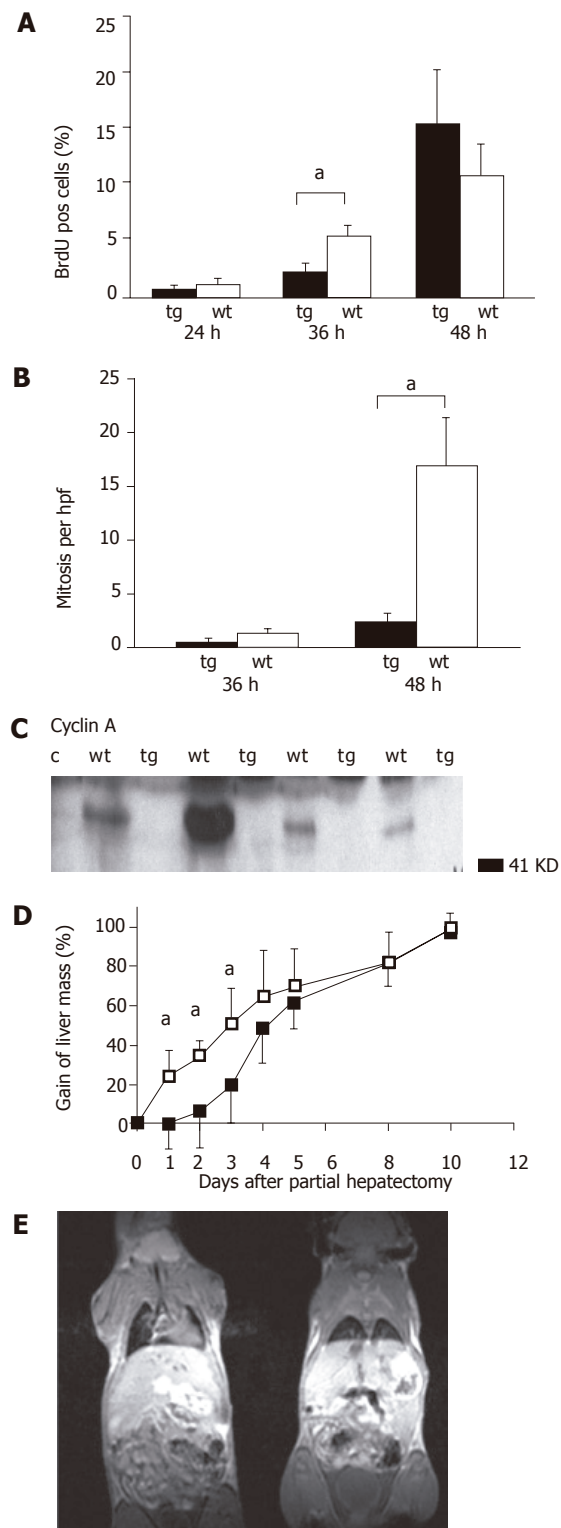


Figure 1 Retarded liver regeneration in dnFADD mice. **A:** Onset of DNA synthesis was delayed in transgenic animals; after 36 h of PH, the amount of BrdU-positive cells was significantly smaller compared to wild-type animals, while the numbers did not differ significantly later on; **B:** Showing a markedly higher number of mitosis per high power field in wild-type animals after 48 h ($^*P<0.05$); **C:** Cyclin A protein expression was not detectable in transgenic animals whereas, detectable in wild-type animals at 36 h after hepatectomy. **D:** Onset of liver mass restoration was retarded in transgenic animals ($^*P<0.05$) but surviving mice reached around 90% of the pre-operative volume as did wild-type animals. The average immediate postoperative liver volumes in the wild-type and transgenic group were 49% and 48% of the preoperative liver volume, respectively. **E:** MRI scans were used to determine liver mass during regeneration.

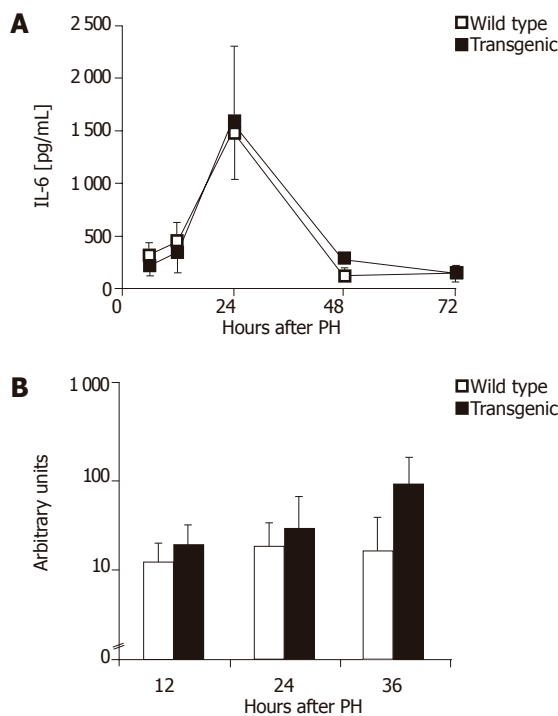


Figure 2 Expression of IL-6 in transgenic hepatocytes. **A:** Serum IL-6 levels peaked around 24 h after partial hepatectomy. There was no difference between wild-type and transgenic animals; **B:** After 36 h of PH, transgenic animals showed significant higher IL-6 mRNA expression in whole liver lysate as compared to wild-type animals, measured by Light Cycler analysis.

regeneration.

High mortality after partial hepatectomy in *dnMORT1/FADD* mice

Surprisingly, we observed significantly higher early postoperative mortality after resection of 70% of the liver mass among the transgenic mice expressing the dominant negative MORT1/FADD mutant (Figure 3) compared to the wild-type littermates. We found a significant difference in postoperative mortality during the first 24 h, when 16.49% (13/81) of transgenic animals whereas, only 2.77% (2/72) of wild-type mice died ($P < 0.01$, Fisher's exact test). However, the mortality did not significantly differ later on. In order to elucidate the cause of different mortality rate, we measured liver enzymes during the early phase of regeneration. However, we did neither detect significant differences in liver function tests (Figure 4) nor differences in the degree of steatosis in liver sections at 36 or 48 h after PH (data not shown).

DISCUSSION

In the present study, we could show for the first time that MORT1/FADD acts as a multi-functional adaptor protein in the liver *in vivo*. In addition to its critical role in CD95- and CD120a-mediated liver failure^[9], MORT1/FADD is involved in initiating liver regeneration upon PH. These results underline the dual role of MORT1/FADD, i.e., it was originally identified as an adaptor protein which

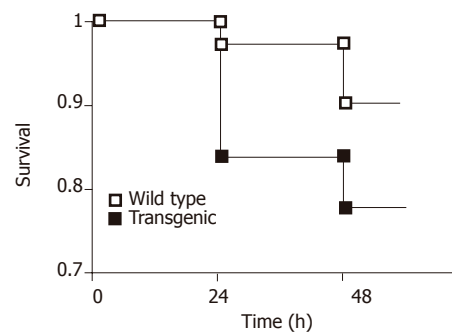


Figure 3 Differences in postoperative mortality. Within the first 24 h after PH, transgenic mice expressing the dominant negative FADD mutant showed a significant higher postoperative mortality ($P < 0.01$). Within the first 24 h, 13 out of 81 transgenic mice died whereas only 2 out of 72 wild-type mice died. At later time points, no difference in postoperative mortality was detected.

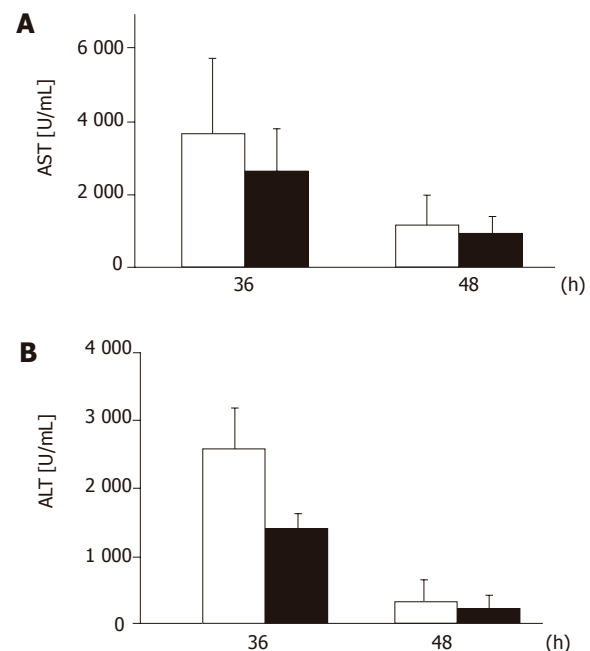


Figure 4 Liver enzymes test. Serum AST (left panel) and ALT (right) levels in wild-type and transgenic mice were not significantly different at 36 or 48 h after PH.

is pivotal to convey death signals of the death receptors CD95 and CD120a^[3,4] and the TRAIL receptors DR4 and DR5^[18]. Initial evidence that the axis MORT1/FADD-caspase-8 has an additional role beside mediating apoptotic cell death revealed in the experiments with MORT1/FADD and caspase-8 knock out animals, showing that both genetic defects lead to embryonic lethality around d 11 *in utero* with similar pathology of cardiac malformation^[5,6,19]. Interestingly, mice lacking functional flip/cash gene, a caspase-8 homolog without enzymatic activity, also died^[20] *in utero* with a similarly impaired cardiac development. Later on, experiments with T-cells lacking MORT1/FADD or expressing a dominant negative mutant indicated an additional role of MORT1/FADD in

the regulation of cell proliferation^[5-8,21].

The exact mechanism by which MORT1/FADD contributes to cell proliferation is still enigmatic, especially when the involved downstream molecules are not identified. Phosphorylation of MORT1/FADD at position serine 191 in mouse (corresponding to serine 194 in human beings) seems to be of crucial importance^[15,22]. Current data have substantiated the idea of a bifurcation of the signal pathway at the level of MORT1/FADD by either sending a death signal via activation of caspase-8 or signaling towards cell proliferation. FLIP, which is also recruited to the receptor complex, has been reported to modulate caspase-8-mediated cell death and is a good candidate for further delivering a proliferation signal^[23]. Indeed, FLIP has been shown to be involved in T-cell proliferation^[23]. However, it has not been completely understood how FLIP further mediates the proliferation signal leading to the activation of ERK^[23]. The molecular architecture of the intracellular bifurcation is complex, since the lack of caspase-8 does not only provide deficiency in death receptor apoptosis signaling but also confers an impaired T-cells proliferation^[14]. According to data of Iimuro *et al.*^[24] and Chaisson *et al.*^[25], NF- κ B is not involved in the orchestration of the initial DNA synthesis after PH.

A regenerating liver is protected against CD95-induced cell death^[26]. This might partially be attributed to the downregulation of CD95 but resetting the function of proapoptotic molecules, such as MORT1/FADD during the initial proliferative response might also contribute. It is not obvious which receptor engages MORT1/FADD to contribute to liver regeneration. CD120a (TNF-receptor 1) and CD95 are both candidates since TNF-R1 knock-out mice^[27] as well as mice with the decreased hepatic CD95 expression (*lpr*-genotype)^[13] showed a pronounced delay of liver regeneration after PH. In our study, the cause of a high postoperative mortality in transgenic mice remains unclear. We could not find signs of liver dysfunction in the early postoperative phase and there was also presence of IL-6 expression. We even detected higher intrahepatic IL-6 mRNA levels in the transgenic mice. A previous study also reported no difference between wild-type and transgenic animals upon LPS or CpG-DNA challenge^[28].

In conclusion, we provide further evidence for a complex role of the adaptor molecule MORT1/FADD which is also involved in death and proliferation pathways in hepatocytes. This observation should be taken into consideration while targeting anti-apoptotic as well as anti-proliferative therapies.

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Vascular endothelial growth factor and angiopoietins regulate sinusoidal regeneration and remodeling after partial hepatectomy in rats

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Abstract

AIM: To study the regulatory mechanisms of sinusoidal regeneration after partial hepatectomy.

METHODS: We investigated the expression of angiopoietin (Ang)-1, Ang-2, Tie-2, and vascular endothelial growth factor (VEGF) in regenerating liver tissue by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Diagnostics) and also immunohistochemical staining after 70% hepatectomy in rats. In the next step, we isolated liver cells (hepatocytes, sinusoidal endothelial cell (SEC), Kupffer cell, and hepatic stellate cells (HSC)) from regenerating liver tissue by *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these angiogenic factors after hepatectomy. Proliferation and apoptosis of SECs were also evaluated by proliferating cell nuclear antigen (PCNA) staining and the terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labeling (TUNEL) assay, respectively.

RESULTS: VEGF mRNA expression increased with a peak at 72 h after hepatectomy, decreasing thereafter. The expression of Ang-1 mRNA was present at detectable levels before hepatectomy and increased slowly with a peak at 96 h. Meanwhile, Ang-2 mRNA was hardly detected before hepatectomy, but was remarkably induced at 120 and 144 h. In isolated cells, VEGF mRNA expression was found mainly in the hepatocyte fraction. Meanwhile, mRNA for Ang-1 and Ang-2 was found in the SEC and HSC fractions, but was more prominent in the latter. The PCNA labeling index of SECs increased slowly, reaching a peak at 72 h, whereas apoptotic SECs were detected between 120 h and 144 h.

CONCLUSION: Ang-Tie system, together with VEGF, plays a critical role in regulating balance between

SEC proliferation and apoptosis during sinusoidal regeneration after hepatectomy. However, the VEGF system plays a more important role in the early phase of sinusoidal regeneration than angiopoietin/Tie system.

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Key words: Vascular endothelial cell growth factor; Angiopoietin; Sinusoidal endothelial cell; Hepatectomy; Liver regeneration

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INTRODUCTION

The regenerative capacity of the liver is typically triggered by hepatic injuries, including partial hepatectomy or hepatocyte loss caused by viral or chemical injury^[1-3]. Liver regeneration is a highly complex and organized process, but extensive studies have revealed that a number of cytokines and growth factors are involved in the regulation of hepatocyte proliferation^[4-6]. Nonparenchymal cells (Kupffer cells, sinusoidal endothelial cells (SECs)) have both stimulatory and inhibitory influences on hepatocyte replication though a paracrine manner after hepatectomy^[7]. As a consequence of hepatocyte proliferation, remodeling of the sinusoids has to be carried out for the supply of blood flow to the newly replicating hepatocytes in the process of liver regeneration, most probably with penetration of SECs into hepatocyte clusters. Presumably, SECs proliferate and assemble into tubes with cell-cell connections, followed by recruitment of hepatic stellate cells (HSCs) and Kupffer cells, to restore normal sinusoidal architecture. Lindahl *et al.*^[8] reported that during vascular development and remodeling, endothelial cells initially proliferate, and recruit mural cell (pericytes or smooth muscle cells) precursors via secretion of platelet-derived growth factor-BB, and endothelial cell-mural cell contact is essential for vessel stabilization^[9]. On the other hand, Martinez-Hernandez *et al.*^[10] suggested that during liver regeneration, the laminin-containing Ito cells initially extend cell processes into the

hepatocyte clusters, followed by SECs, to form new sinusoids after hepatectomy, but at present the precise steps involved in sinusoidal regeneration remain poorly understood. However, recent studies including ours showed that vascular endothelial growth factor (VEGF) greatly contributes to the proliferation of SECs via up-regulated VEGF receptors during liver regeneration^[11-15]. Moreover, we have clearly shown that newly replicated hepatocytes that are devoid of sinusoids are the major source of VEGF after hepatectomy^[13]. However, the detail mechanisms of sinusoidal regeneration and remodeling are still open to discussion.

Recently, the angiopoietin (Ang)-Tie system has been identified as a second family of endothelial cell-specific growth factors^[16-18], and has been reported to act in a complementary and coordinated fashion with VEGF, thus playing an important role in vascular maturation and remodeling^[17,18]. The modulatory effect of angiopoietins on VEGF-induced angiogenesis is distinct^[19-21], although angiopoietins alone are not mitogenic for endothelial cells *in vitro*^[18], and do not promote neovascularization *in vivo*^[19]. Angiopoietins include both receptor activators (Ang-1) and receptor antagonists (Ang-2)^[18]. Binding of Ang-1 causes auto-phosphorylation of tyrosine kinase Tie-2 receptor, whereas Ang-2 binding conversely suppresses auto-phosphorylation of Tie-2. The Tie-2 signal is, therefore, defined by quantitative balance between Ang-1 and Ang-2 activities^[19,20]. The Ang-1/Tie-2 pathway in the presence of VEGF is thought to mediate the vital functions of vascular stabilization and vascular maturation, via integration of peri-endothelial cells into the vascular wall. In contrast to Ang-1, Ang-2 induces vascular regression in the absence of VEGF but increases vascular sprouting in its presence^[19,21]. Accordingly, it seems to be reasonable to assume that the Ang-Tie system and the VEGF system, participate in the process of sinusoidal regeneration and remodeling after partial hepatectomy.

In this study, we investigated the expression of Ang-1, Ang-2, Tie-2, and VEGF in regenerating liver tissue by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Diagnostics, Mannheim, Germany) and immunohistochemical staining after 70% hepatectomy in rats. In the next step, we isolated liver cells (hepatocyte, SEC, Kupffer cell, and HSC) from regenerating liver tissue by *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these angiogenic factors after hepatectomy. Proliferation and apoptosis of SECs were also evaluated by proliferating cell nuclear antigen (PCNA) staining and the terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labeling (TUNEL) assay, respectively, to determine the putative role of VEGF and angiopoietins, especially their complementary and coordinated effects during sinusoidal regeneration after hepatectomy.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 250 to 300 g, were used with

Table 1 Primer sequences

Gene		Primer sequence	T (°C)
VEGF	Sense	GCA CTG GAC CCT GGC TTT AC	56
	Antisense	CTG CAG GAA GCT CAT CTC TC	
Ang-1	Sense	GTG GCT GGA AAA ACT TGA GA	52
	Antisense	TGG ATT TCA AGA CGG GAT GT	
Ang-2	Sense	GAC CAG TGG GCA TCG CTA CG	56
	Antisense	CTG GTT GGC TGA TGC TAC TG	
Tie-2	Sense	TGC CAC CAT CAC TCA ATA CC	54
	Antisense	AAA CGC CAA TAG CAC GGT GA	
GAPDH	Sense	GGC ATG GAC TGT GGT CAT GAG	56
	Antisense	TGC ACC ACC AAC TGC TTA GC	

T : annealing temperature.

the approval of the Chiba Animal Care Committee. The animals were housed in a temperature- and humidity-controlled environment with a 12-h light dark cycle. The study followed our institution's criteria for care and use of laboratory animals in research, which conform to the National Institutes of Health guidelines. Under ether anesthesia, 70% of the liver was resected as previously described^[22]. The operative procedure was carried out by means of a clean, but not sterile technique. For the assessment of the Ang-1, Ang-2, Tie-2, and VEGF expressions, the right inferior lobe of the liver was carefully excised before and 12, 24, 48, 72, 96, 120, 144, and 240 h ($n = 6$ at each time point) after hepatectomy.

Quantitative RT-PCR analysis of VEGF, angiopoietins, and Tie-2 mRNA expressions

Total RNA was extracted from liver tissues or freshly isolated liver cells by the acid guanidium-thiocyanate/phenol/chloroform method, and 1 mg of extracted total RNA was subjected to a reverse transcription reaction, using Ready To GoTM T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA from 33 ng of total RNA was used as a template. VEGF, Ang-1, Ang-2, and Tie-2 mRNA levels were quantified by a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I. Details of the primers used in this study are summarized in Table 1. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing for 10 s, and extension at 72 °C for 20 s. The expression level of each angiogenic factor was adjusted by the level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as a ratio to GAPDH mRNA. To confirm the specific amplification from the target mRNAs, some of the PCR products of each primer set were directly sequenced.

Liver cell isolation from regenerating liver tissue

In the next step, liver cells were isolated from the regenerating liver tissue at the peak periods of each angiogenic factor expression (at 48 and 72 h for VEGF, at 72 and 96 h for Ang-1, at 120 and 144 h for Ang-2, and at 72 and 96 h for Tie-2) after hepatectomy, to determine potential cellular sources of VEGF, Ang-1, Ang-2, and

Tie-2 mRNA. Rat hepatocytes were isolated according to the methods of Gumucio *et al.*^[23] Briefly, the rat liver was portally perfused first with Mg^{2+}/Ca^{2+} -free Hanks' buffer at 37 °C, and then with Eagle's minimal essential medium (EMEM) containing 0.05% collagenase (Type I, Sigma, St Louis, MO, USA). The liver was excised and placed in a petri dish containing fresh EMEM with 0.05% collagenase. Glisson's capsule was stripped, and cells were released by gentle manipulation. The resulting crude preparation was filtered through 250-µm and 100-µm nylon meshes. Hepatocytes were then separated by differential centrifugation. Cell viability and purity were >95%. The SECs, Kupffer cells, HSCs were also isolated by *in situ* collagenase perfusion and counterflow elutriation, as described by Knook *et al.*^[24] with minor modifications. A JE-5.0 elutriator rotor (Beckman Instruments, Palo Alto, CA, USA) was used in a J6-MI Beckman centrifuge. The separation process was started by adding the nonparenchymal cell suspension to a sample-mixing chamber. The HSCs were eluted at a flow rate of 16 to 18 mL/min, and at a speed of 3 200 r/min. The SECs were then eluted at a flow rate of 23 to 26 mL/min, and Kupffer cells at a flow rate of 36 to 39 mL/min, and at a speed of 2 500 r/min. The purity of HSCs was >92%, as assessed 24 h after seeding by their typical light-microscopic appearance and their positive immunofluorescent staining for desmin^[25]. The purity of SECs and Kupffer cells was >95%, >90%, respectively, as assessed 24 h after seeding by diacyllow-density lipoprotein incorporation^[26], and positive immunofluorescent staining for ED-1^[27]. To evaluate the expression of Ang-1, Ang-2, Tie-2, and VEGF mRNA in each cell fraction, freshly isolated cells were used for total RNA extraction.

Immunohistochemical examination of Ang-1, Ang-2 and Tie-2

For immunohistochemical examination of Ang-1, Ang-2, and Tie-2, the liver tissues excised after hepatectomy were fixed overnight in 2% paraformaldehyde solution at 4 °C, embedded in tissue-tek compound (Miles Laboratories, Elkhart, IN, USA), and frozen in liquid nitrogen for preparation of cryostat sections. Sections (6 µm) were stained with Ang-1, Ang-2, and Tie-2 antibodies (dilution: 1:100, respectively, Santa Cruz Biotechnology Inc. Delaware, CA, USA) and incubated overnight at 4 °C. Immunostained cells were detected out using LSAB kit (Dako, Copenhagen, Denmark) according to the manufacturer's instructions. All sections were then lightly counterstained with 0.1% hematoxylin.

PCNA labeling index

Immunohistochemical staining for PCNA was performed on formalin-fixed and paraffin-embedded liver tissue with anti-PCNA antibody as previously described^[28, 29]. The three-step immunoperoxidase method using strept-avidin biotin complex (Dako, Copenhagen, Denmark) was performed, according to the procedure described by Hall *et al.*^[29]. PC-10 monoclonal antibody (Dako, Copenhagen,

Denmark) was used at a dilution of 1:100 and incubated overnight at 4 °C. PC-10 immunostaining was evaluated based on the percentage of positive nuclei of 100 SECs at high power (400x), and expressed as a PCNA labeling index. In this study, the spindle-shaped sinusoid-lining cells in the open sinusoids at high power (400x) were regarded as SECs^[30].

TUNEL assay

Regenerating liver tissue was evaluated using the TUNEL assay to identify SECs undergoing apoptosis. Portions of the liver were fixed with 4% paraformaldehyde and embedded in paraffin. Six micrometer thick sections were prepared, and stained by the TUNEL method using an *in situ* apoptosis detection kit (Takara Biomedicals Co., Osaka, Japan) according to the manufacturer's instructions. Each stained section was examined at high power fields (200 x), and TUNEL-positive cells in sinusoids were evaluated.

Statistical analysis

The results were expressed as mean±SD. Statistical analysis was made by the Mann-Whitney test for unpaired data. $P<0.05$ was considered significant.

RESULTS

Changes expression of VEGF, angiopoietin, and Tie-2 mRNA in regenerating liver tissue

The expression of VEGF mRNA was at very low levels before hepatectomy. However, VEGF mRNA in the regenerating liver tissue started to increase 24 h after hepatectomy, with a peak at 72 h. A significant increase was found at 48 and 72 h when compared to the prehepatectomy value ($P<0.05$, respectively). Thereafter, VEGF mRNA returned to baseline levels at 120 h (Figure 1A). The changes in Ang-1, Ang-2, and Tie-2 mRNA expressions are shown in Figure 1B. Ang-1 mRNA was present at detectable levels even before hepatectomy, and increased after 48 h of hepatectomy, with a peak at 96 h. A significant increase was seen at 72 and 96 h ($P<0.05$ vs 0 h, respectively). Meanwhile, Ang-2 mRNA was hardly detectable before hepatectomy, but was significantly up-regulated at 120 and 144 h ($P<0.03$ vs 0 h, respectively). The expression of Tie-2 mRNA was low before hepatectomy, but tended to increase between 72 h and 120 h after hepatectomy.

VEGF, angiopoietin, and Tie-2 mRNA in specific cell populations isolated from regenerating liver tissue

To determine the cellular sources of VEGF, Ang-1, Ang-2, and Tie-2 mRNA in the regenerating liver tissue, four types of liver cells were isolated at key time points: at 48 and 72 h for VEGF, at 72 and 96 h for Ang-1, at 120 and 144 h for Ang-2, at 72 and 96 h for Tie-2, because the expression of each was strongly induced at the indicated time points after hepatectomy (Figure 1). The results are shown in Figure 2. In cell isolates, the mRNA for VEGF was found mainly in the hepatocyte fraction, but in other

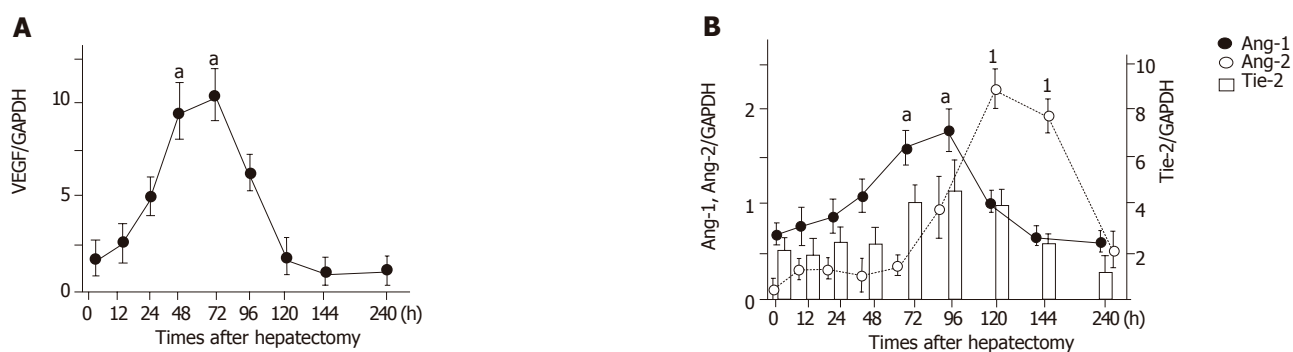


Figure 1 Changes in vascular endothelial growth factor (VEGF), angiopoietin (Ang) and Tie-2 mRNA expressions after 70% hepatectomy. **A:** Quantification of VEGF mRNA levels after hepatectomy by RT-PCR using a LightCycler (Roche

Diagnostics). ^a $P < 0.05$ vs 0 h; **B:** quantification of Ang-1 (closed circle), Ang-2 (open circle), and Tie-2 (open bar) mRNA levels after hepatectomy by RT-PCR using a LightCycler. ^a $P < 0.05$ vs 0 h, ¹ $P < 0.03$ vs 0 h, respectively.

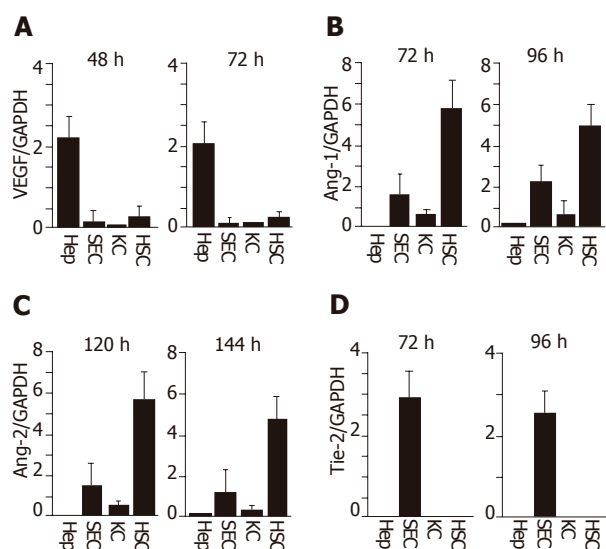


Figure 2 Expressions of VEGF (A), Ang-1 (B), Ang-2 (C) and Tie-2 mRNA (D) in isolated liver cells at different time points.

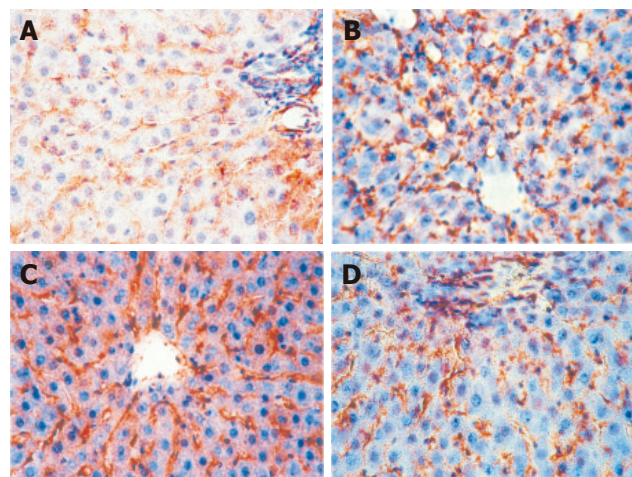


Figure 3 Immunohistochemical staining for angiopoietins (Ang) and Tie-2 protein. Ang-1 protein before hepatectomy (A), 96 h after hepatectomy (B), Ang-2 protein induction 120 h after hepatectomy (C), Tie-2 protein before hepatectomy (D) (original magnification $\times 200$).

cell fractions, VEGF mRNA was hardly expressed. On the other hand, the mRNA for Ang-1 and Ang-2 was found mainly in the SEC and HSC fractions, but Ang-2 mRNA expression was more prominent in the latter. The mRNA for Tie-2 was found to be in the SEC fraction alone.

Immunohistochemical staining for angiopoietins and Tie-2

Ang-1 protein was detectable along sinusoids even before hepatectomy (Figure 3A), and was upregulated at 96 h after hepatectomy (Figure 3B). Meanwhile, Ang-2 protein was not detected before hepatectomy, but was remarkably up-regulated along sinusoids at 120 h after hepatectomy (Figure 3C). The Tie-2 protein was observed along sinusoids even before hepatectomy (Figure 3D).

PCNA labeling index and TUNEL staining

The PCNA labeling index was less than 5% in SECs before hepatectomy. However, a slow increase in the PCNA labeling index of SECs was observed after 24 h

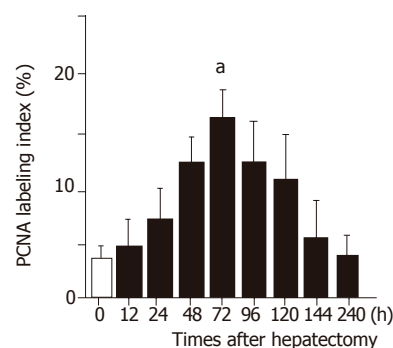


Figure 4 PCNA labeling index of sinusoidal endothelial cells (SECs) after 70% hepatectomy in rats. ^a $P < 0.05$ vs 0 h.

of hepatectomy, reaching a peak of 16% at 72 h ($P < 0.05$ vs 0 h) after hepatectomy (Figure 4). Figure 5 shows TUNEL staining of the regenerating liver at 120 h after hepatectomy. Before hepatectomy, apoptotic cells in

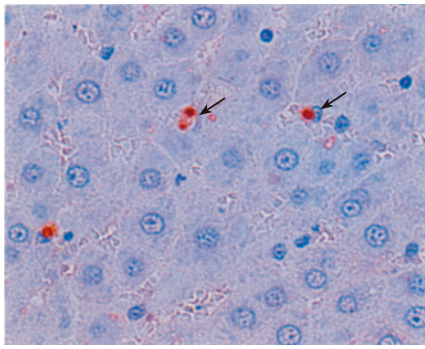


Figure 5 TUNEL staining of regenerating liver tissue at 120 h after hepatectomy. Arrows indicate TUNEL stained-positive sinusoidal cells.

sinusoids were uncommon in the liver, and even fewer such cells were detected within 72 h of hepatectomy. However, a few sinusoidal cells were found to be TUNEL stain-positive between 120 h and 144 h after hepatectomy.

DISCUSSION

In the present study, we showed that VEGF expression in the remnant liver started to increase 24 h after hepatectomy, with a peak at 72 h, which coincided with SEC proliferation, as assessed by PCNA staining. Moreover, in cell isolates from the regenerating liver tissue, VEGF mRNA expression was found mainly in the hepatocyte fraction, suggesting that VEGF secreted from hepatocytes may greatly contribute to SEC proliferation in a paracrine manner. Thereafter, Ang-1 and Ang-2 mRNA expression in the remnant liver increased, but the Ang-2 peak was much later than the Ang-1 peak. In isolated cells, both Ang-1 mRNA and Ang-2 mRNA were found to be abundant in nonparenchymal cells, especially in HSCs. Furthermore, apoptosis of SECs was only detected at 120 and 144 h after hepatectomy, which overlapped with elevated levels of Ang-2. Viewed from the standpoint of Ang-1 function as previously reported in angiogenesis, increased expression of Ang-1 might be related with maturation of sinusoids, presumably characterized by cell-cell contact between SECs and surrounding cells. Meanwhile, elevated Ang-2 in the absence of VEGF induction might be associated with the apoptotic changes in superfluous SECs for cessation of the regenerative process. On the other hand, their receptor, Tie-2 was constitutively expressed on SECs, and tended to increase in the later phase of regeneration, synchronized with up-regulation of Ang-1 and Ang-2. Altogether, these results strongly suggest that angiopoietins released from nonparenchymal cells may largely contribute to the later phase of sinusoidal regeneration and remodeling via Tie-2 receptor after hepatectomy.

In the present study, Ang-1 was constitutively expressed in the liver at the mRNA level and also at the protein level, but VEGF mRNA and Ang-2 mRNA were hardly detectable before hepatectomy. These results might be associated with the maintenance of stability and the

maturation of existing sinusoids in the steady state of the liver. Meanwhile, Ang-2 mRNA expression was largely induced in the late phase of regeneration after hepatectomy. Furthermore, apoptotic SECs were detected between 120 h and 144 h, suggesting that SEC apoptosis might be associated with elevated levels of Ang-2 in the absence of VEGF induction. Since apoptosis is known to be a strategic biologic process of eliminating unnecessary cells under certain physiological conditions^[31], the SEC apoptosis observed in this study might be related to the presence of superfluous SECs in the late phase of regeneration. Greene *et al.*^[32] recently demonstrated that SECs play an important role in the regulation of regenerating hepatic mass by means of proliferation and apoptosis. Therefore, apoptosis of SECs in the late phase of regeneration in this study might be associated with cessation of the regenerative process of the liver.

In cell isolates from the regenerating liver tissue, Ang-2-produced cells were found to be nonparenchymal cells, mainly HSCs. HSCs are known to be located in the space of Disse, below the sinusoidal endothelial cell lining, in close to and partially intercalated between hepatocytes, with their long processes extending along sinusoids^[33]. Under physiological conditions, HSCs embrace the sinusoids as “liver-specific pericytes”^[34]. At present, the factors that initiate HSC activation are not yet fully understood, although platelet-derived growth factor and transforming growth factor- β (TGF- β) may play a key role in HSC activation and proliferation^[35]. On the other hand, TGF- β , a potent inhibitor of hepatocyte proliferation has been reported to increase significantly in the later phase of regeneration, and is known to be mainly released from sinusoidal cells after 70% hepatectomy in rats^[36-38]. That is, it would be reasonable to expect activation and proliferation of HSCs in a late phase of regeneration, and subsequent release of several growth factors, including angiopoietins, as well as induction of extracellular matrix, after hepatectomy.

In this study, we focused on the role of angiopoietins and VEGF, especially their complementary and coordinated role in the process of sinusoidal regeneration after hepatectomy, and clearly demonstrated that the Ang-Tie system, together with VEGF, played a critical role in regulating the balance between SEC proliferation and apoptosis during sinusoidal regeneration and remodeling after partial hepatectomy. Accordingly, it is clear that production of VEGF and angiopoietins in the liver must be coordinated both quantitatively and temporally to ensure appropriate sinusoidal regeneration after hepatectomy. However, the VEGF system plays a more important role in the early phase of sinusoidal regeneration than the angiopoietin/Tie system. In addition, close communications between different types of liver cells are clearly important in proceeding through these complex steps of regeneration. However, more detail studies are required to elucidate the regulatory mechanisms of sinusoidal regeneration and remodeling, which are only understood at a descriptive level at present, and

also to provide clues for treatment of impaired hepatic regeneration after major hepatectomy, from this point of view.

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• BASIC RESEARCH •

Clinical value of rapid urine trypsinogen-2 test strip, urinary trypsinogen activation peptide, and serum and urinary activation peptide of carboxypeptidase B in acute pancreatitis

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Abstract

AIM: To assess the usefulness of urinary trypsinogen-2 test strip, urinary trypsinogen activation peptide (TAP), and serum and urine concentrations of the activation peptide of carboxypeptidase B (CAPAP) in the diagnosis of acute pancreatitis.

METHODS: Patients with acute abdominal pain and hospitalized within 24 h after the onset of symptoms were prospectively studied. Urinary trypsinogen-2 was considered positive when a clear blue line was observed (detection limit 50 µg/L). Urinary TAP was measured using a quantitative solid-phase ELISA, and serum and urinary CAPAP by a radioimmunoassay method.

RESULTS: Acute abdominal pain was due to acute pancreatitis in 50 patients and turned out to be extrapancreatic in origin in 22 patients. Patients with acute pancreatitis showed significantly higher median levels of serum and urinary CAPAP levels, as well as amylase and lipase than extrapancreatic controls. Median TAP levels were similar in both groups. The urinary trypsinogen-2 test strip was positive in 68% of patients with acute pancreatitis and 13.6% in extrapancreatic controls ($P < 0.01$). Urinary CAPAP was the most reliable test for the diagnosis of acute pancreatitis (sensitivity 66.7%, specificity 95.5%, positive and negative predictive values 96.6% and 56.7%, respectively), with a 14.6 positive likelihood ratio for a cut-off value of 2.32 nmol/L.

CONCLUSION: In patients with acute abdominal pain, hospitalized within 24 h of symptom onset, CAPAP in serum and urine was a reliable diagnostic marker of acute pancreatitis. Urinary trypsinogen-2 test strip showed a clinical value similar to amylase and lipase. Urinary TAP was not a useful screening test for the diagnosis of acute pancreatitis.

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Key words: Acute pancreatitis; Urinary trypsinogen-2; Urinary trypsinogen activation peptide; Activation peptide of carboxypeptidase B; Acute abdominal pain

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INTRODUCTION

Most patients with acute pancreatitis have mild and self-limited disease that resolves spontaneously, but about 20% of attacks are severe, with a mortality of about 10-25%^[1]. Early diagnosis of acute pancreatitis is crucial to ensure rapid and appropriate treatment. However, the clinical features of acute pancreatitis can be difficult to distinguish from those of other acute abdominal conditions, and the diagnosis may be overlooked at first. Determination of amylase in serum or urine is the principal laboratory method for diagnosing acute pancreatitis. However, hyperamylasemia may be absent in 19% of cases^[2], and increases in pancreatic enzyme levels can occur in patients with acute abdominal pain of extrapancreatic in origin^[3-5]. The pathophysiology of acute pancreatitis involves the activation of the pancreatic proenzymes. The activation of trypsinogen followed by the activation of other pancreatic zymogens occurs early in the course of pancreatitis, in proportion to the extent of pancreatic injury^[6]. Concentrations of the activation peptide of trypsinogen (TAP) in urine and of the activation peptide

of procarboxypeptidase B (CAPAP) in urine and serum have shown to be promise predictors of severity in acute pancreatitis^[7,8]. However, the clinical value of these markers for the early diagnosis of acute pancreatitis is unclear. Until now, a previous study of urinary TAP levels^[7] and three other studies of serum and urine CAPAP^[9-11] have shown significantly higher concentrations of these peptides in acute pancreatitis compared to non-pancreatic abdominal pain, therefore, suggesting the usefulness of these assays for diagnosing acute pancreatitis. On the other hand, qualitative rapid urine trypsinogen-2 test strip is easy to perform and has been shown to be a reliable and useful screening test for acute pancreatitis in daily practice^[12-16], particularly in healthcare units lacking laboratory facilities. This study was conducted to assess the usefulness of urinary trypsinogen-2 test strip, urinary TAP, and serum and urine CAPAP levels compared with conventional enzymes (serum amylase and lipase) for diagnosing acute pancreatitis.

MATERIALS AND METHODS

Patients

All consecutive patients with acute abdominal pain admitted to the Section of Gastroenterology of an Acute-care University Hospital in Alicante, Spain, in an 8-month period, participated in an observational, prospective, cohort study. Admission to the hospital within 24 h of symptom onset was the criterion for inclusion. The diagnosis of acute pancreatitis was based on (1) typical clinical symptoms and at least a threefold increase of serum amylase, once other causes of abdominal pain had been excluded, and (2) evidence of pancreatic inflammation by imaging studies and/or surgery. The severity of acute pancreatitis was assessed according to the criteria established at the International Symposium on Acute Pancreatitis in Atlanta in 1992^[17]. According to our protocol, a CT scan was performed in patients with severe acute pancreatitis, with unknown etiology and whenever it was necessary to establish the diagnosis of acute pancreatitis. In all the patients, blood and urine samples were collected within the first 24 h of hospitalization. The Hospital General Universitario Ethic's Committee approved the protocol, and all patients gave their informed consent for the inclusion in the study. Patients were divided into two groups according to the origin of acute abdominal disease. There were 50 patients with acute pancreatitis (25 men, 25 women, mean age 62.5 ± 16.5 years) and 22 patients (11 men, 11 women, mean age 50.8 ± 22.4 years) whose acute abdominal pain turned out to be extrapancreatic in origin. A CT scan was performed in all the 22 patients with non-pancreatic abdominal pain to rule out the possibility of acute pancreatitis.

Laboratory tests

Serum amylase and lipase concentrations were measured by routine methods of our laboratory and for the remaining diagnostic markers, urine and serum samples were frozen

immediately after collection and stored at (-20 °C) until analysis.

Serum amylase concentrations were measured using an enzymatic assay (Amyl, Boehringer Mannheim Systems, Mannheim, Germany). For this assay the limit of detection is 3 U/L and the range of the standard curve was comprised between 3 and 1 200 U/L. The reference range of serum amylase, previously established in our laboratory, was 26-100 U/L. Serum lipase was measured using a colorimetric enzymatic technique (Lip, Boehringer Mannheim Systems, Mannheim, Germany). For this assay the limit of detection was 3 U/L and the range of the standard curve was comprised between 3 and 300 U/L. The reference range of serum lipase, previously established in our laboratory, was 13-60 U/L.

Serum and urine CAPAP concentrations were measured using a radioimmunoassay method (CAPAP RIA kit, Euro-Diagnostica, Malmö, Sweden). The kit is based on a competitive radioimmunoassay using antibodies against human CAPAP. CAPAP both in standards and samples compete with ¹²⁵I-labeled CAPAP in binding to the antibodies. The ¹²⁵I-CAPAP binds to the antibodies in an inverse proportion to the concentrations of CAPAP both in standards and samples. Antibody-bound ¹²⁵I-CAPAP is separated from the unbound fraction using the double antibody solid-phase technique. The radioactivity of the pellets was then measured. For this assay, the lower limit of detection was about 0.4 nmol/L and the range of the standard curve was comprised between 0 and 20 nmol/L. When the results above this limit were obtained, serum samples were diluted at 1:5 and urine samples at 1:10 were processed again.

Urinary TAP was measured using a quantitative solid-phase ELISA based on the competition between free and immobilized peptide binding to an antibody to TAP (TAPKIT, Biotrin International Ltd, Dublin, Ireland). Urine samples were collected in sterile containers with EDTA (at final concentration 5-10 nmol/L) and diluted to a ratio of 1:4 in the assay buffer according to the manufacturer's instructions. TAP linked to a carrier protein is immobilized on the solid phase to which the peptide calibrator or diluted (1:4) urine sample, plus the TAP antibody, was added. After the reaction, the amount of antibody bound to the solid phase was measured spectrophotometrically (450 nm) after sequential addition of rabbit antibody to IgG-biotin conjugate, streptavidin-horseradish peroxidase, and tetramethyl-benzidine. The lower detection limit was 0.2 nmol/L (0.8 nmol/L in the 1:4 diluted samples) and the range of the standard curve was comprised between 0.14 and 200 nm.

The Actim Pancreatitis test strip (Medix Biochemica, Finland) for urinary trypsinogen-2 is an immunochromatographic test. After the test strip has been dipped into the urine sample, trypsinogen-2 is bound to monoclonal antibody-labeled blue latex particles, which migrate across a nitrocellulose membrane with a zone containing another antibody specific for another epitope on trypsinogen-2. At trypsinogen-2 concentrations higher than 50 µg/L, a blue line develops

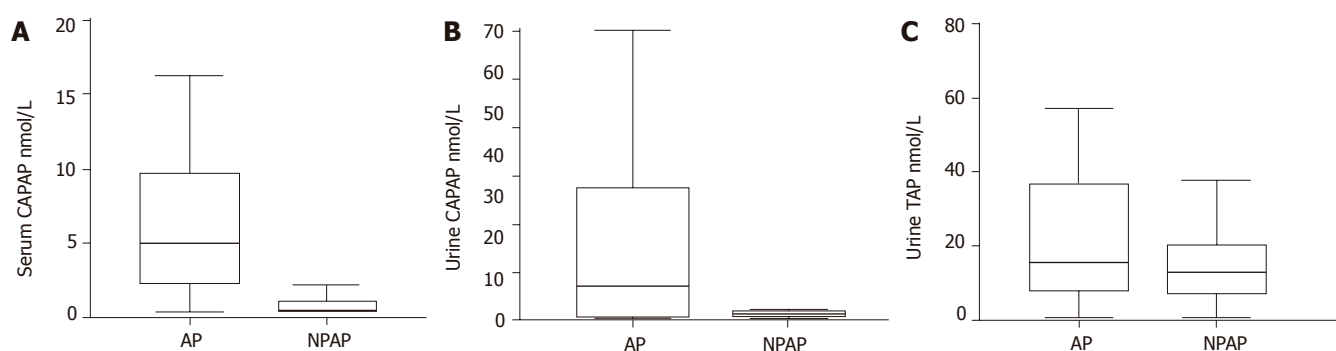


Figure 1 **A:** CAPAP concentration in serum within 24 h of hospital admission in patients with acute pancreatitis and in extrapancreatic controls. Data as median and interquartile range (AP, acute pancreatitis; NPAP, non-pancreatic abdominal pain); **B:** CAPAP concentration in urine within 24 h of hospital admission in patients with acute pancreatitis and in extrapancreatic controls. Data as median and interquartile range (AP: acute pancreatitis; NPAP, non-pancreatic abdominal pain); **C:** Urinary TAP levels within 24 h of hospital admission in patients with acute pancreatitis and in extrapancreatic controls. Data as median and interquartile range (AP: acute pancreatitis; NPAP, non-pancreatic abdominal pain).

Table 1 Clinical and outcome data in 50 patients with acute pancreatitis

	Number of patients	Percentage
Male/female	25/25	
Age, yr, mean (SD)	62.5 (16.5)	
Etiology		
Gallstones	30	60
Idiopathic	12	24
Alcohol	3	6
Post-ERCP	3	6
Hypertriglyceridemia	2	4
Severity		
Severe	15	30
Mild	35	70
Complications		
Local	12	24
Systemic	17	34
Death	5	10

ERCP, endoscopic retrograde cholangiopancreatography.

Table 2 Etiology in 22 controls with non-pancreatic acute abdomen

Etiology	Number of patients	Percentage
Acute cholecystitis	5	22.7
Acute appendicitis	3	13.6
Colicky pain	3	13.6
Intestinal obstruction	2	9.1
Active Crohn's disease	2	9.1
Acute cholangitis	1	4.5
Perforated peptic ulcer	1	4.5
Gastric carcinoma	1	4.5
Epiploic appendicitis	1	4.5
Abdominal abscess	1	4.5
Bleeding peptic ulcer	1	4.5
Mesenteric lymphadenitis	1	4.5

off values that were selected. The SPSS/PC+10 statistical package on a personal computer was used for the analysis of data.

in this zone. The test was considered positive when a clear blue line was detected within 5 min. A control line was used to indicate proper functioning of the strip. If the control line was undetectable the assay was repeated.

Statistical analysis

Descriptive data were given as median with interquartile range (25th and 75th percentiles). The Mann-Whitney *U* test was used to compare the results between patients with acute pancreatitis and extrapancreatic controls. Statistical significance was set at $P < 0.05$. Receiver operating characteristics (ROC) curves of serum and urine CAPAP levels and urinary TAP concentrations were used to determine the optimal cut-off levels. For serum concentrations of amylase and lipase, threefold increases in the reference values recommended by our laboratory were selected as cut-off values. Using these cut-off points, the sensitivity, specificity, positive and negative predictive values and positive likelihood ratio in establishing the diagnosis of acute pancreatitis were calculated. Comparison of different prognostic markers was made using the positive likelihood ratio for the different cut-

RESULTS

Of the 50 patients with acute pancreatitis, 15 (10 men and 5 women, mean age 66 ± 16 years) had severe disease and 35 (17 men and 18 women, mean age 61 ± 17 years) had mild disease. Etiology of pancreatitis and outcome are shown in Table 1. Local pancreatic complications included necrosis in 7 patients, pseudocyst in 4 and abscess in 1. Systemic complications consisted of renal failure in 6 patients, respiratory insufficiency in 6 and gastrointestinal hemorrhage in 5. Death occurred in 5 of 15 patients with severe pancreatitis. Diagnoses established in controls with non-pancreatic acute abdomen are shown in Table 2.

As compared with extrapancreatic controls, patients with acute pancreatitis had significantly higher values of amylase [942.20 (316.25 - $1\,333.42$) U/L *vs* 52 (29.50 - 91.20) U/L, $P < 0.001$] and lipase [$1\,084$ (228.75 - $2\,133.75$) U/L *vs* 22 (15.50 - 39) U/L, $P < 0.001$] in serum. On the other hand, patients with acute pancreatitis showed significantly higher values of CAPAP in the serum and urine than controls with non-pancreatic acute abdomen, i.e., CAPAP in serum 5.05 (2.23 - 10.04) nmol/L *vs* 0.40 (0.40 - 1.14) nmol/L, $P < 0.001$;

Table 3 Diagnostic accuracy of cut-off values of CAPAP in serum and urine and urinary TAP to distinguish severe acute pancreatitis from the mild form of the disease

Proenzyme, cut-off value within 24 h of admission	Pre-test probability %	Sensitivity %	Specificity %	PPV %	NPV %	Positive likelihood ratio	Post-test probability %
Amylase serum, >330 U/L	69.4	74	86.4	92.5	59.3	5.4	92.4
Lipase serum, >180 U/L	69.4	84	85.7	93.4	72	5.87	93
CAPAP serum, >1.53 nmol/L	69.4	85	90.9	95.2	74	9.3	95
CAPAP urine, >2.32 nmol/L	69.4	66.7	95.5	96.9	56.7	14.6	97
TAP urine, >10.01 nmol/L	69.4	68.8	40	73.3	34.7	1.13	71.9
Trypsinogen-2 urine, positive	69.4	68	86.4	91.9	54.3	5	91.9

PPV, positive predictive value; NPV, negative predictive value.

CAPAP in urine 7.05 (0.93-29.5) nmol/L *vs* 1.36 (0.80-1.85) nmol/L, $P < 0.01$) (Figures 1A and B). However, there were no statistically significant differences in urinary TAP between patients and controls [15.45 (7.79-37.05) nmol/L *vs* 12.70 (7.03-20.27) nmol/L] (Figure 1C). The urinary trypsinogen-2 test strip was positive in 68% of patients with acute pancreatitis and 13.6% in extrapancreatic controls ($P < 0.01$).

Sensitivity, specificity, predictive values, pre-test probability, post-test probability and positive likelihood ratio for the different cut-off values of CAPAP in serum and urine, urinary TAP and trypsinogen-2 test are shown in Table 3. Urinary CAPAP was the most reliable test for the diagnosis of acute pancreatitis (sensitivity 66.7%, specificity 95.5%, positive predictive value 96.6%, negative predictive value 56.7% and post-test probability 97%), with a 14.6 positive likelihood ratio for a cut-off value of 2.32 nmol/L.

DISCUSSION

This study demonstrates that CAPAP in serum and urine (especially urinary CAPAP) was a reliable diagnostic marker of acute pancreatitis providing slightly better results than serum amylase and lipase. Urinary trypsinogen-2 test strip showed a clinical value similar to amylase and lipase. Urinary TAP was not a useful screening test for the diagnosis of acute pancreatitis. In agreement with previous studies^[18,19], we also found that serum amylase and lipase were adequate diagnostic markers of acute pancreatitis.

In respect to urinary TAP concentrations, the present results are consistent with other studies^[20,21] in which this marker was not useful for diagnosing acute pancreatitis. However, in the study of Neoptolemos *et al.*^[7], there were statistically significant differences in urinary TAP levels between patients with acute pancreatitis and controls both at 24-48 h of hospital admission and 24-48 h after the onset of symptoms. It may be possible that the increase in urinary TAP levels at 48 h compared to 24 h and the high rate of alcoholic pancreatitis included in the study of Neoptolemos *et al.*^[7] may account for the differences observed with the present findings.

With regard to clinical value of serum and urine CAPAP concentrations, both assays were reliable early markers for diagnosing acute abdominal pain of pancreatic origin. These results are similar to previous findings by our group in a series of 22 patients^[22] and confirm that this marker of acute pancreatitis is capable of both simultaneously

diagnosing and assessing severity of disease at the time of admission to the hospital^[8-11]. Certainly, we do not know why urinary TAP was not a useful screening test for the diagnosis of acute pancreatitis whereas urinary CAPAP levels were a reliable test, since both assays indicate an activation of pancreatic zymogens. Probably, the different kinetics of release and techniques of determination of both markers could explain this point. More studies are necessary to assess it. On the other hand, laborious and time consuming work are pitfalls of current CAPAP assays for rapid screening in a routine diagnostic setting. Moreover, it hardly improves the diagnostic accuracy of the routine markers (amylase and lipase).

Rapid urine trypsinogen-2 strip test has been introduced in an effort to decrease the number of misdiagnosed cases of acute pancreatitis in an emergency setting. In the present study, the test was positive in 68% of patients with acute pancreatitis and 13.6% in extrapancreatic controls, with a sensitivity of 68% and specificity of 86.4%. These results are slightly less favorable than those reported in other series^[12-16], in which 90% sensitivity was almost reached. However, it should be noted that a high percentage of patients with non-pancreatic acute abdominal pain with a clinical profile similar to acute pancreatitis (cholecystitis five cases, biliary colic three cases) were included in our study. Therefore, the present results far from decreasing the clinical usefulness of urinary trypsinogen-2 test strip, contribute to delimit the true value of this rapid screening test in clinical practice. On the other hand, it has to be considered that the inclusion process of patients with abdominal pain in this investigation may be biased, since patients admitted to our department have been pre-selected by the emergency department, and this likely justifies the high proportion of patients with AP in our series (50/72).

In summary, in patients with acute abdominal pain, hospitalized within 24 h of symptom onset, CAPAP in serum and urine was a reliable diagnostic marker of acute pancreatitis, but it provided only slightly better results than serum amylase and lipase and, although we have not made a detailed economical analysis, it seems not to be cost-effective to use it as a routine diagnostic marker. On the other hand, urinary trypsinogen-2 test strip showed a clinical value similar to amylase and lipase. Urinary TAP was not a useful screening test for the diagnosis of acute pancreatitis.

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Lymphocytic colitis: A clue to bacterial etiology

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Abstract

AIM: To find out the role of bacteria as a possible etiological factor in lymphocytic colitis.

METHODS: Twenty patients with histopathological diagnosis of lymphocytic colitis and 10 normal controls were included in this study. Colonoscopic biopsies were obtained from three sites (hepatic and splenic flexures and rectosigmoid region). Each biopsy was divided into two parts. A fresh part was incubated on special cultures for bacterial growth. The other part was used for the preparation of histologic tissue sections that were examined for the presence of bacteria with the help of Giemsa stain.

RESULTS: Culture of tissue biopsies revealed bacterial growth in 18 out of 20 patients with lymphocytic colitis mostly *Escherichia coli* (14/18), which was found in all rectosigmoid specimens (14/14), but only in 8/14 and 6/14 of splenic and hepatic flexure specimens respectively. In two of these cases, *E coli* was associated with proteus. Proteus was found only in one case, Klebsiella in two cases, and *Staphylococcus aureus* in one case. In the control group, only 2 out of 10 controls showed the growth of *E coli* in their biopsy cultures. Histopathology showed rod-shaped bacilli in the tissue sections of 12 out of 14 cases with positive *E coli* in their specimen's culture. None of the controls showed these bacteria in histopathological sections.

CONCLUSION: This preliminary study reports an association between *E coli* and lymphocytic colitis, based on histological and culture observations. Serotyping and molecular studies are in process to assess the role of *E coli* in the pathogenesis of lymphocytic colitis.

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Key words: Lymphocytic colitis; *E coli*; Histopathology; Culture

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INTRODUCTION

Microscopic colitis is a syndrome consisting of chronic watery diarrhea, a normal or near normal gross appearance of the colonic mucosa, and a specific histological picture described as either lymphocytic or collagenous colitis^[1]. The term microscopic colitis was first introduced by Read *et al.*^[2] that was found in eight patients who were having idiopathic chronic diarrhea and normal colonoscopic findings. The diagnosis of microscopic colitis in such patients was based on the presence of excess amount of chronic inflammatory cells in the lamina propria of colorectal mucosa. A closely related term "lymphocytic colitis" was introduced by Lazenby *et al.*^[3], who reported that the most distinctive histological feature of microscopic colitis is the presence of an excess amount of intraepithelial lymphocytes, and renamed microscopic colitis as "lymphocytic colitis". Recently, the histopathology of lymphocytic colitis has been more specified by Lamps and Lazenby^[4].

The etiology of lymphocytic colitis is still unclear, and several factors have been claimed. First, it is induced by drugs especially non-steroidal-anti-inflammatory drugs (NSAIDs)^[5]. However, subsequent studies found that there is no association between NSAID consumption and microscopic colitis^[6]. Vascular tonics have also been suspected to play a part in the pathogenesis of lymphocytic colitis via chronic activation of the mucosal immune system by one or several components of such drugs^[7]. Other drugs that claimed to be a cause of lymphocytic colitis include Lansoprazole^[8] and Modopar^[9]. The second concept is the reported association of microscopic colitis with celiac disease, which may indicate a common pathogenesis^[1,10,11]. Nevertheless, many patients with celiac disease do not show colonic lymphocytosis^[12]. Lastly it is the possible role of pathogenic organisms. Some investigators have found spirochetes in some patients with microscopic colitis, and suggested that these microorganisms are capable of inducing the disease^[13]. Conversely, earlier studies reported that spirochetes have no clinical significance^[14]. In a search for infectious causes, Afzalpurkar *et al.*^[15] investigated 14 patients with chronic idiopathic diarrhea by stool examination and stool culture as well as a culture of the jejunal fluid. Although stool examination and culture were negative, abnormal growth

of bacteria in the jejunal fluid was noted in four patients. Similarly, the culture of the jejunal aspirate in 14 patients having chronic idiopathic diarrhea has revealed bacterial growth in one patient who was successfully treated with antibiotics^[2].

The aim of the current preliminary study was to search for a possible role of the pathogenic bacteria in lymphocytic colitis. This has been achieved by thorough histologic examination as well as culture of the colonic tissue specimens, which is to the best of our knowledge not previously reported in the literature.

MATERIALS AND METHODS

Patients

The present study consisted of 20 patients fulfilling the classic definition of lymphocytic colitis, which is described as a triad of idiopathic chronic, watery diarrhea, normal or nearly normal colonoscopic findings, and colonic epithelial lymphocytosis without a thickened subepithelial collagen band^[16]. The patients were chosen from the in- and outpatients of Ain-Shams University Hospital during the period between 1999 and 2001, after excluding all other causes of diarrhea by a thorough clinical examination, radiologic, endoscopic, and laboratory investigations. The exclusion criteria were autoimmune diseases, any systemic disease that could cause diarrhea as diabetes, history of food sensitivity, use of laxatives or other drugs that could cause diarrhea, presence of ova and parasites or occult blood in stools.

Ten age- and sex-matched healthy volunteers with no diarrhea or any other gastrointestinal diseases were used as negative controls. The endoscopic and histologic pictures of their colonic mucosae were normal. All patients and controls were not allowed to take antibiotics for 3 wk before the biopsy, since antibiotic treatment may affect the bowel microbial flora. Informed written consent was obtained from all the patients and volunteers.

Stool analysis

Microscopic examination of fresh unstained smears was done to exclude parasitic infestation.

Colonoscopic examination and biopsy

Using lower CF 100 L video colonoscope (Olympus), the whole colon was examined for any pathological lesions including the inspection of the terminal ileum if possible. Patients were selected on the basis of having normal or nearly normal colonoscopic findings. From each patient and control, four tissue samples were taken from each of the following sites: hepatic flexure (HF), splenic flexure (SF), and rectosigmoid region (RS). These biopsy specimens were collected separately, then each specimen was divided into two parts, the first part was put in normal saline and sent for culture, the other part was fixed in 10% buffered formalin and sent for histopathology.

Culture studies of biopsy samples

For all subjects, fresh colonic tissue specimens obtained

from HF, SF, and RS regions were incubated separately on the media listed in Table 1 to detect bacterial flora. Specimens were crushed before incubation. The isolated intestinal bacteria were identified by colony morphology, microscopic examination of gram stained smears, conventional biochemical reactions and API20 E identification system (BioMerieux) for the identification of Gram-negative bacteria.

Histopathology

Formalin-fixed colonic tissue specimens obtained from the 20 patients and 10 controls (3 specimens from each subject) were processed separately for the preparation of paraffin blocks. The latter were sectioned at 5 μ m and stained with hematoxylin and eosin (H&E) for routine examination, Masson trichrome for the demonstration of subepithelial collagen band, and modified Giemsa stain to search for bacteria (as followed in the cases of chronic gastritis) to detect *Helicobacter pylori*^[17]. One pathologist (T.E.H.) examined all the tissue sections without the knowledge of the clinical or endoscopic findings. All 60 specimens were examined for the histologic criteria of lymphocytic colitis, which were modified by Lazenby *et al.*^[3]. These criteria were simplified as follows. (1) Surface epithelial damage, which was seen as flattened or syncytial appearance of surface cells instead of the tall columnar cells that are normally observed. (2) Crypt distortion. Each of these two findings was graded according to severity into 1-3 scales (1+: mild, 2+: moderate, and 3+: marked). (3) Mononuclear cellular infiltrate in the lamina propria. This was graded into 1-4 scales (1+: normal, 2+: mild, 3+: moderate, and 4+: marked). (4) Intraepithelial lymphocytes. It was scored as the number of lymphocytes per 100 surface epithelial cells. It is worth mentioning that any case showing histologic features other than lymphocytic colitis was excluded from the study. These histologic exclusion criteria included features suggestive of inflammatory bowel disease^[18], features of collagenous colitis^[19], presence of ova or parasites, presence of viral inclusions, and melanosis coli.

Statistical analysis

Fisher's exact test was used to compare the patients and controls regarding the frequency of the bacterial growth.

Chi-square test was used to assess the association between the presence of bacteria and the severity of the histopathological features.

RESULTS

Clinical data

Patients with lymphocytic colitis included in the study consisted of 15 males and 5 females with a male to female ratio of 3:1. Ages ranged 20-67 years, with a mean of 36 ± 10.3 years. All patients presented with chronic watery diarrhea of unknown cause for a period of 1-4.5 years with a mean of 2.5 ± 0.76 years. The daily motions ranged 3-7 times (mean 4.5 ± 1.3 times). All patients had associated mild to moderate abdominal discomfort. Five patients

Table 1 Culture media used for the detection of bacterial flora in colonic biopsies

Medium	Temperature	Incubation time (h)	Organism
MacConkey's agar	37 °C	48-72	Coliform bacilli (<i>E coli</i> and <i>Klebsiella</i>) and <i>Proteus</i>
Aerobic blood agar	37 °C	24-48	<i>Staphylococcus aureus</i>
Anaerobic blood agar	37 °C	24-48	Anaerobic bacteria as <i>Bacteroides</i> spp.
Microaerophilic Campy-blood agar	42 °C	24-48	<i>Campylobacter jejuni</i>
MacConkey's selective medium	25 °C	24-48	<i>Yersinia Enterocolitica</i>
Selenite broth enrichment medium and Salmonella-Shigella agar	37 °C	24	<i>Salmonella</i> and <i>Shigella</i>

Table 2 Culture results of 60 colonic tissue specimens from 20 patients with lymphocytic colitis

Organism	Patients (n = 20)	RS specimens (n = 20)	SF specimens (n = 20)	HF specimens (n = 20)
<i>E coli</i>	12	12	8	6
<i>E coli</i> + <i>Proteus</i>	2	2	0	0
<i>Proteus</i>	1	1	1	0
<i>Klebsiella</i>	2	2	2	1
<i>Staphylococcus aureus</i>	1	1	1	1
Total number	18	18	12	20

RS: rectosigmoid, SF: splenic flexure, HF: hepatic flexure.

Table 3 Histopathologic data of 60 colonic biopsies from 20 patients with lymphocytic colitis

Feature	RS	SF	HF
1 Surface epithelial damage			
1+	8	10	8
2+	6	6	6
3+	6	4	6
2 Crypt distortion			
1+	17	16	17
2+	3	4	3
3+	0	0	0
3 LP Inflammation			
2+	5	6	2
3+	11	10	12
4+	4	4	6
4 Mean % of IEL	23	22	28

RS: rectosigmoid, SF: splenic flexure, HF: hepatic flexure, LP: lamina propria, IEL: intraepithelial lymphocytes.

had significant weight loss. No other gastrointestinal manifestations were found.

Culture results

Bacterial growth was observed in the colonic tissue specimens of 18 out of the 20 patients (90%). The most frequent isolated organism was *E coli*, which was demonstrated in 14 out of 18 culture positive cases (77.8%). These organisms were seen in the rectosigmoid specimens in all the 14 cases, splenic flexure growth was found in 8 of them, and hepatic flexure growth in 6 cases only. In 2 out of the 14 cases, *E coli* was associated with the growth of *Proteus*. Other isolated bacteria were *Klebsiella* (two cases) and *Staphylococcus aureus* (one case). Other organisms such as *Shigella*, *Salmonella*, *Campylobacter*, *Yersinia* did not grow on their specific media. In the controls, *E coli* was obtained from the rectosigmoid samples of two cases (20%). The frequency of the bacterial growth was significantly higher in the patients than in the controls ($P = 0.0003$) (Table 2).

Histopathological results

The most prominent feature in all 60 colonic biopsies obtained from the 20 patients was the increased number of mononuclear cells in the lamina propria (Figure 1A) observed in all the three regions examined (rectosigmoid, splenic, and hepatic flexures), indicating total colitis. The inflammatory cells were mainly lymphocytes with less number of plasma cells and histiocytes; few polymorphonuclear cells were present in two cases with no crypt abscess formation. A small number of eosinophils were seen in three patients. Epithelial damage was mild to moderate in the majority of colonic biopsies (Figure 1A). The average crypt distortion was mild to moderate in all colonic biopsies. Few lymphocytes could be seen between the crypt epithelial cells, but not in the crypt lumen (Figure 1B). No other inflammatory cells were present in the lumen or epithelial cells of the crypts.

The percentage of the inflammatory cells in the surface epithelium ranged 15-50% with a mean of 23%, 22%, and 28% in the rectosigmoid, splenic flexure and hepatic flexure, respectively. The intraepithelial inflammatory cells were lymphocytes only (Table 3 and Figure 1C).

In 12 out of 18 patients with bacterial overgrowth in their colonic tissue specimens, rod-shaped bacilli were seen in the histologic tissue sections obtained from various colonic sites. These bacilli were visible on H&E-stained sections, but were more easily demonstrated by Giemsa stain. They were closely applied to the surface epithelium (Figure 1D), or within the lumen of the crypts which showed destruction of their lining cells (Figure 1E). It is important to note that the intercellular and intracellular localization of the organisms could not be assessed because these modes of colonization could not be definitely identified by light microscopy alone. The presence of bacteria was significantly associated with the degree of epithelial damage ($\chi^2 = 9.9$, $P = 0.007$) and the degree of the cellular infiltrate in the lamina propria

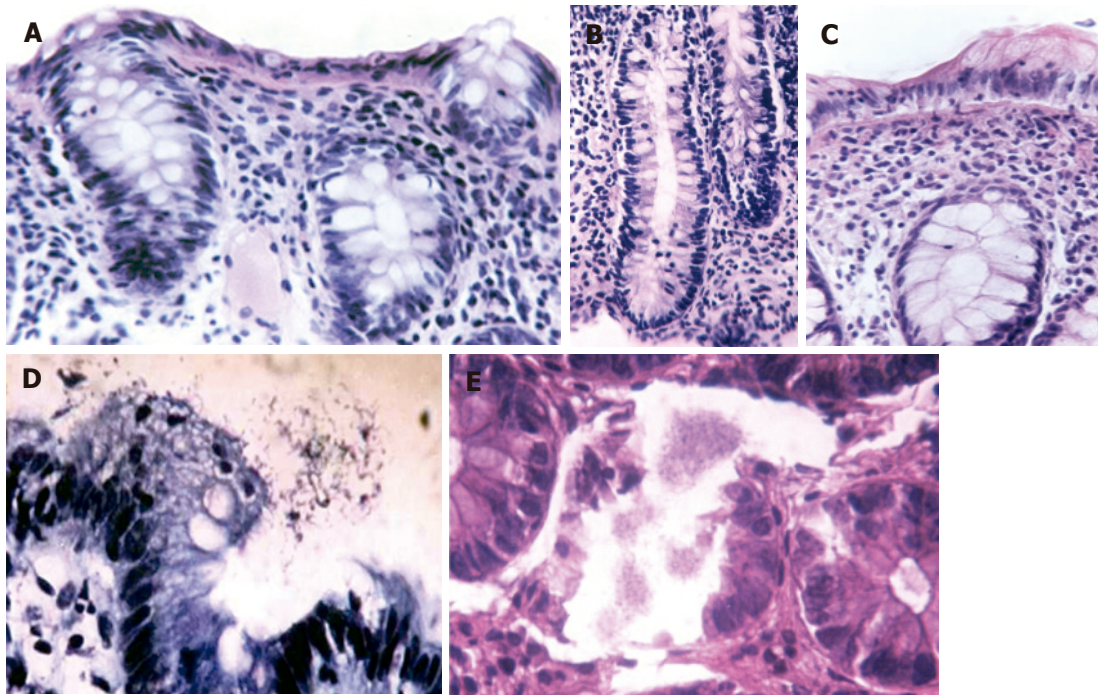


Figure 1 Cases of lymphocytic colitis showing respectively the damage of surface epithelium and increased mononuclear cells in lamina propria (H&E $\times 250$) (A), few lymphocytes between crypt epithelial cells (H&E $\times 250$) (B), intraepithelial lymphocytes (H&E $\times 250$) (C), rod-shaped bacilli adherent to the surface epithelium (Giemsa stain $\times 400$) (D), and bacilli within the lumen of transverse section of crypt (H&E $\times 400$) (E).

($\chi^2 = 6.0$, $P = 0.05$). No significant relationship could be obtained between the presence of bacteria and the degree of crypt distortion (Table 4).

In all 30 colonic biopsies obtained from the 10 healthy controls, the colonic mucosa was nearly normal with no visible bacilli by H&E and Giemsa staining.

DISCUSSION

The main purpose of the present preliminary study was to investigate the possible role of bacteria in the pathogenesis of lymphocytic colitis. To achieve this aim, patients with definite diagnosis of lymphocytic colitis and normal controls were subjected to colonoscopy and biopsy. Specimens were examined in two ways. First, histopathological examination, including a thorough search for bacteria, similar to that for *H. pylori* in gastric biopsies. Second, culture of colonic tissue specimens on specific media to isolate bacterial growth. Although some authors have investigated the possible role of infection in lymphocytic colitis or chronic idiopathic diarrhea, their methodology is limited to stool culture and/or culture of the jejunal fluid^[2,15,19,20]. To the best of our knowledge, this is the first study, which attempted to search for bacteria in the colonic biopsies in cases of lymphocytic colitis by histologic examination of tissue sections as well as culture of the colonic tissue.

Microscopically, we could identify small or large number of rod-shaped bacilli closely related to the surface of the colonic mucosa and within the lumen of the crypts in 12 out of 14 patients with positive *E. coli* in biopsy culture.

Table 4 Correlation between bacteria in tissue sections and histopathologic features in 60 colonic biopsies from 20 patients with lymphocytic colitis

Feature	Bacteria		χ^2	P
	+	-		
1 Epithelial damage				
1+	1	25	9.9	0.007
2+	4	14		
3+	7	9		
2 Crypt distortion				
1+	10	40	0.4	NS
2+	2	8		
3+	0	0		
3 LP Inflammation				
2+	2	11	6.0	0.05
3+	4	29		
4+	6	8		

LP: lamina propria, NS: not significant.

It seems that *E. coli* was most likely seen in the histologic sections of our tissue specimens, because these bacilli were seen in patients with positive *E. coli* in biopsy culture.

The demonstration of these bacilli in the colonic mucosa of our patients with lymphocytic colitis raises several questions. The first question is why they have not been seen before in colonic tissue sections of such cases? We think that these bacteria have not been previously demonstrated because Giemsa stain is not routinely applied for colonic mucosal biopsy specimens and bacteria are easily overlooked on H&E-stained sections. The second question to be raised is whether these bacteria

are pathogenic or merely commensals in the colonic mucosa. From our point of view, we suggest that these bacilli, which have been demonstrated histologically, are pathogenic rather than commensals, since they are closely related to the mucosal surface and within the lumen of crypts, and not within the tissue debris. Moreover, they are always associated with prominent colonic pathology. It is worthy of notion that we could not demonstrate such bacteria in all colonic biopsies obtained from the healthy controls.

The relation between lymphocytic colitis and bacterial infection is still controversial. Gebbers and Laissue^[13] reported the presence of spirochetes in some patients with microscopic colitis, and suggested that these microorganisms are capable of inducing the disease. On the other hand, earlier studies reported that spirochetes have no clinical significance^[14]. Perk *et al.*^[21] presented a patient who had chronic watery diarrhea and showed stool culture positive for *Campylobacter jejuni*. Examination of her colonic biopsies led to the diagnosis of lymphocytic colitis. The authors believed that the patient's disease is due to an infectious process. Also, Tremaine^[22] reported that bismuth subsalicylate is effective in the treatment of some patients with lymphocytic colitis, a finding that supports the concept of infectious etiology in such disease.

It is interesting to note that the search for bacterial etiology in cases of chronic watery diarrhea has been expanded to include the type of bacteria responsible for this disease. The most common organism investigated in this regard is *E coli*^[23-25].

Diarrheagenic *E coli* (*E coli* causing diarrhea) is divided into four groups: enteropathogenic (causing acute diarrhea in infants and children), enteroinvasive (causing Shigella-like dysentery), enterohemorrhagic (causing hemorrhagic colitis and bloody diarrhea) and enterotoxigenic (causing travelers' diarrhea)^[26]. In addition to these groups, two other types, which were previously classified as enteropathogenic, have been recently identified. The first is enteroaggregative *E coli* (EaggEC), which is now considered as a cause of prolonged diarrhea^[27]. Another recently described type of *E coli* is the diffusely adherent type which has been found to be significantly associated with chronic diarrhea^[28]. It is important to note that many strains of *E coli* belonging to the classic enteropathogenic type are now found to be enteroaggregative by DNA hybridization or PCR. This indicates that routine culture studies and even serotyping are of limited value for the identification of various groups of diarrheagenic *E coli*^[29].

These new types of *E coli* may be more likely the types seen in the tissue sections and biopsy culture of our patients, being associated with chronic diarrhea as mentioned above, and may be related to the pathogenesis of lymphocytic colitis, since it can colonize both in large and in small intestines. In a study by Kang *et al.*^[30], strains of enteroaggregative *E coli* isolated from patients with diarrhea resulted in the colonization of both small and large intestines. In support to this suggestion is the study of Afzalpurkar *et al.*^[15], who found that 4 out of 14 patients with chronic idiopathic diarrhea have abnormal

growth of bacteria in aspirated jejunal fluid and the results of quantitative cultures are consistent with contamination. In addition, one of the four patients had steatorrhea, which is one of the hallmarks of bacterial overgrowth syndrome. These data have led the authors to speculate that *E coli* or other bacteria not identified by routine culture methods are involved in the etiology of chronic idiopathic diarrhea. It is worth mentioning that the lack of response to antibiotics in the four patients with bacterial overgrowth reported in the previous study should not be considered as evidence against bacterial etiology, since multiple drug resistance in EaggEC has been reported by some studies recommending quinolone treatment for EaggEC-associated diarrhea^[31].

In conclusion, bacteria play a possible role in lymphocytic colitis in which rod-shaped bacilli adherent to the colonic mucosa can be seen as in cases of *Helicobacter* gastritis. Further studies are now in process to investigate the pathogenicity of these organisms in case of lymphocytic colitis.

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Effects of ethanol on antioxidant capacity in isolated rat hepatocytes

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CONCLUSION: These results suggest that long-time incubation with higher concentration of ethanol (100 mmol/L) decreased the cell viability by means of reducing GRD and CAT activities and increasing lipid peroxidation.

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Key words: Hepatocyte; Ethanol; Lipid peroxidation; Antioxidant capacity

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Abstract

AIM: To investigate dose-response and time-course of the effects of ethanol on the cell viability and antioxidant capacity in isolated rat hepatocytes.

METHODS: Hepatocytes were isolated from male adult Wistar rats and seeded into 100-mm dishes. Hepatocytes were treated with ethanol at concentrations between 0 (C), 10 (E10), 50 (E50), and 100 (E100) mmol/L (dose response) for 12, 24, and 36 h (time course). Then, lactate dehydrogenase (LDH) leakage, malondialdehyde (MDA) concentration, glutathione (GSH) level, and activities of glutathione peroxidase (GPX), glutathione reductase (GRD), superoxide dismutase (SOD), and catalase (CAT) were measured.

RESULTS: Our data revealed that LDH leakage was significantly increased by about 30% in group E100 over those in groups C and E10 at 24 and 36 h. The MDA concentration in groups C, E10 and E50 were significantly lower than that in group E100 at 36 h. Furthermore, the concentration of MDA in group E100 at 36 h was significantly higher by 4.5- and 1.7-fold, respectively, than that at 12 and 24 h. On the other hand, the GSH level in group E100 at 24 and 36 h was significantly decreased, by 32% and 28%, respectively, compared to that at 12 h. The activities of GRD and CAT in group E100 at 36 h were significantly less than those in groups C and E10. However, The GPX and SOD activities showed no significant change in each group.

INTRODUCTION

Ethanol is metabolized to acetaldehyde by some enzymes in the body, including alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP 2E1), catalase (CAT), xanthine oxidase (XO), etc. Then acetaldehyde is decomposed to acetic acid by acetaldehyde dehydrogenase (ALDH) in mitochondria. Several studies have provided evidences for reactive oxygen species (ROS) generation during ethanol metabolism, including superoxide radical^[1], hydrogen peroxide^[2], hydroxyl radical^[3], and 1-hydroxyethyl radical^[4]. Numerous studies have indicated that excessive ethanol intake induces the mass production of free radicals in the body, which are considered to be associated with alcoholic liver disease^[5]. Furthermore, a number of experimental studies demonstrated that either acute or chronic alcohol administration to experimental animals increases the formation of lipid peroxidation products, such as malondialdehyde (MDA), and decreases tissue levels of antioxidants, such as glutathione (GSH), in the liver^[6]. Bailey and Cunningham also indicated that the exposure of hepatocytes to ethanol resulted in increased production of ROS, which correlated with decreased cell viability^[7]. The impairment of cellular antioxidant defenses along with the formation of oxygen-derived radicals has been proposed to play a role in causing oxidative damage associated with alcoholic liver disease. Moreover, the individual differences in animals always makes the results inconsistent; therefore, the purpose of this study was to investigate and clarify the influences of different concentrations of ethanol on the

cell viability, antioxidant capacity, and antioxidant enzymes activities in the isolated liver parenchymal cells during different incubation times.

MATERIALS AND METHODS

Preparation of isolated rat hepatocytes

Hepatocytes were isolated from rats according to the two-step collagenase perfusion technique described by Berry and Friend^[8]. Isolated cells were cultured as monolayers in William's medium E with 5% fetal bovine serum and 1 μ mol/L dexamethasone at a density of 1×10^5 cells/mL. After 24 h of incubation at 37 °C in 50 mL/L CO₂, hepatocytes were treated with ethanol at concentrations between 0 (C), 10 (E10), 50 (E50), and 100 (E100) mmol/L (dose response) for 12, 24, and 36 h (time course). Then, the cells were collected using a scraper and resuspended in Tris buffer (50 mmol/L Tris-HCl, 5 mmol/L EDTA, and 1 mmol/L DTT, pH 7.5) for analysis as follows.

Lactate dehydrogenase (LDH) leakage

The viability of hepatocytes was expressed as the percentage of LDH leakage, which was the LDH activity in the culture medium relative to the total LDH activity including the culture medium and cytosolic fraction. The LDH level was determined using the method described by a previous study^[9].

Lipid peroxidation of hepatocytes

The MDA concentration of hepatocytes was assessed colorimetrically at 586 nm using a commercial kit (Calbiochem 437634; Calbiochem-Novabiochem, La Jolla, CA, USA). The concentration was expressed as nmol/mg protein in hepatocytes.

Glutathione (GSH) concentration of hepatocytes

The concentration of reduced GSH in hepatocytes was measured spectrophotometrically at 400 nm using a commercial kit (Calbiochem 354102; Calbiochem-Novabiochem). The concentration was expressed as nmol/mg protein in hepatocytes.

Glutathione peroxidase (GPX) activity of hepatocytes

GPX activity of hepatocytes was determined with a commercial kit (RS 504; Randox Laboratories, Antrim, UK). Twenty microliters of the diluted sample was added to 1 mL of mixed substrate (4 mmol/L GSH, 0.5 U/L GRD and 0.34 mmol/L NADPH dissolved in 50 mmol/L phosphate buffer, pH 7.2, 4.3 mmol/L EDTA). Forty microliters of cumene hydroperoxide (diluted in deionized water) was added to the mixture and GPX activity was measured at 37 °C on a Hitachi U-2000 Spectrophotometer at 340 nm for 3 min. The activity was expressed as mU/mg protein in hepatocytes.

Glutathione reductase (GRD) activity of hepatocytes

GRD activity of hepatocytes was measured with a commercial kit (Calbiochem 359962; Calbiochem-Novabiochem). Two hundred microliters of the diluted

sample was added to 400 μ L of 2.4 mmol/L GSSG buffer (dissolved in 125 mmol/L potassium phosphate buffer, pH 7.5, 2.5 mmol/L EDTA). Four hundred microliters of 0.55 mmol/L NADPH (dissolved in deionized water) was added to the mixture and GRD activity was measured at 340 nm for 5 min on a Hitachi U-2000 Spectrophotometer. The activity was expressed as mU/mg protein in hepatocytes.

Superoxide dismutase (SOD) activity of hepatocytes

SOD activity of hepatocytes was measured with a commercial kit (SD 125; Randox Laboratories). Fifty microliters of the diluted sample was added to 1.7 mL of mixed substrate (50 μ mol/L xanthine and 25 μ mol/L 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride, INT). Two hundred and fifty microliters of XO was added to the mixture and SOD activity was measured at 37 °C on a Hitachi U-2000 Spectrophotometer at 505 nm for 3 min. The activity was expressed as U/mg protein in hepatocytes.

Catalase (CAT) activity of hepatocytes

CAT activity of hepatocytes was determined at 25 °C with Hitachi U-2000 Spectrophotometer UV-VIS Spectrophotometer according to the previous study^[10]. Diluted sample was added to 59 mmol/L H₂O₂ (dissolved in 50 mmol/L potassium phosphate buffer, pH 7.0) and CAT activity was measured at 240 nm for 3 min. One unit of CAT activity was defined as the mmol of H₂O₂ degraded/min/mg protein. The activity was expressed as U/mg protein in hepatocytes.

Total protein concentration

In order to express the antioxidant enzymes activities per gram of protein, total protein concentration of hepatocytes was determined colorimetrically by using a Bio-Rad DC protein assay kit (Cat. No. 500-0116; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Values were expressed as mean \pm SD. To evaluate the differences between the groups studied, two-way analysis of variance (ANOVA) with Fisher's *post hoc* test was used. The SAS software (Vers. 8.2, SAS Institute Inc., Cary, NC, USA) was used to analyze all the data. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Effect of ethanol on LDH leakage

LDH leakage into the culture media of hepatocyte was used to assess the hepatotoxicity of ethanol (Figure 1). LDH leakage in group E10 was nearly the same as that in group C at 12, 24, and 36 h. In contrast, LDH leakage in group E100 was significantly increased by about 30% more than that in groups C and E10 at 24 and 36 h ($P < 0.05$). Furthermore, the LDH leakage showed dose-dependent correlation with the ethanol concentrations ($P = 0.0026$). However, there was no significant correlation between LDH leakage and incubation time of ethanol ($P > 0.05$).

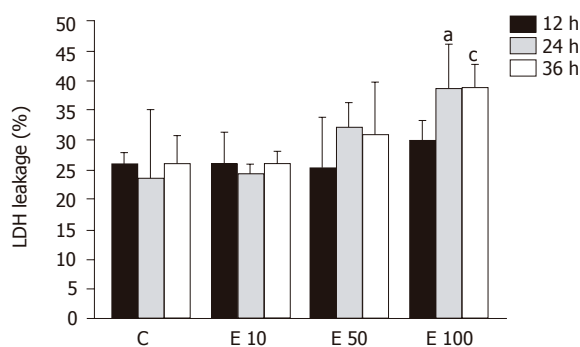


Figure 1 Lactate dehydrogenase (LDH) leakage of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a P <0.05 vs 24 h of group C and E 10; ^c P <0.05 vs 36 h of groups C and E 10.

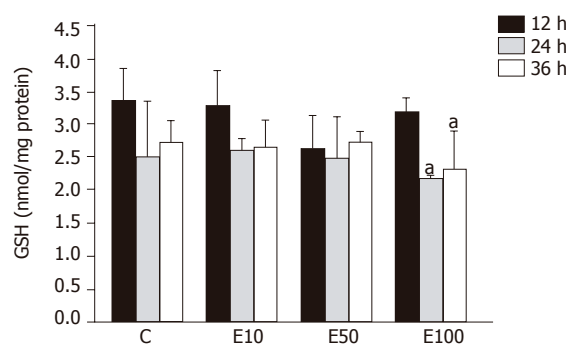


Figure 3 Glutathione (GSH) concentrations of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a P <0.05 vs 12 h of group E 100.

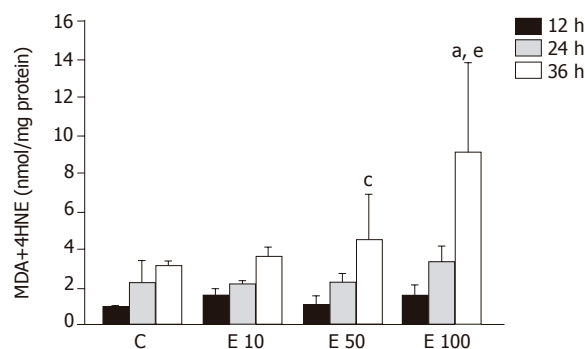


Figure 2 Lipid peroxidation of primary rat hepatocytes in each group. Lipid peroxidation was estimated by the measurement of MDA. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a P <0.05 vs 36 h of groups C, E 10 and E 50; ^c P <0.05 vs 12 h of group E 50; ^e P <0.05 vs 12 h and 24 h of group E 100.

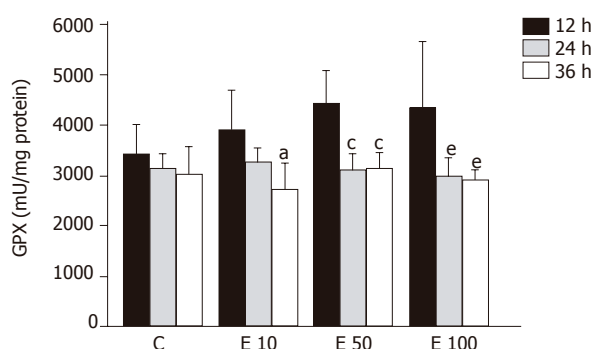


Figure 4 Glutathione peroxidase (GPX) activities of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a P <0.05 vs 12 h of group E 10; ^c P <0.05 vs 12 h of group E 50; ^e P <0.05 vs 12 h of group E 100.

Effect of ethanol on lipid peroxidation

The level of MDA in group E100 at 36 h was significantly increased by 1.9-, 1.5-, and 1.0-fold, respectively, over that in groups C, E10, and E50 (P <0.05, Figure 2). In group E50 and group E100, the lipid peroxidation product was significantly elevated at 36 h (P <0.05), by 2.9- and 4.5-fold, respectively, when compared to that at 12 h. In addition, there was no significant difference in group C and group E10. The level of MDA also showed significant correlation with the incubation concentration and time of ethanol (P = 0.0105 and P = 0.0001, respectively).

Effect of ethanol on antioxidant level

There was no significant difference across the groups at different times (Figure 3). However, in group E100, the GSH level was significantly decreased at 24 and 36 h, by 32% and 28%, respectively, over that at 12 h (P <0.05).

Effect of ethanol on antioxidant enzymes activities

There was no significant difference in GPX activity in each group at different times (Figure 4). However, in group

E10, the GPX activity was significantly decreased at 36 h from that at 12 h (P <0.05). Furthermore, both in groups E50 and E100, GPX activities were significantly lowered at 24 and 36 h from those at 12 h (P <0.05). As a result, the activity of GPX was significantly correlated with the incubation time course (P = 0.0004).

After 36-h incubation, there were significantly fewer GRD activities in groups E50 and E100 than in groups C and E10 (P <0.05, Figure 5). However, both in group C and group E10 at 36 h, the activities of GRD were significantly increased over those at 12 and 24 h (P <0.05). On the other hand, the relation of GRD activity and the incubation time showed positive correlation (P = 0.0002).

There were no significant differences among the groups at different times in SOD activity (Figure 6). SOD activity of group E50 was significantly decreased at 36 h, by 45%, from that at 12 h (P <0.05). Furthermore, in group E100, the SOD activities were significantly decreased at 24 and 36 h, by 40% and 38%, respectively, from that at 12 h (P <0.05). In addition, the activity of SOD exhibited the reverse correlation with the incubation time (P = 0.0018).

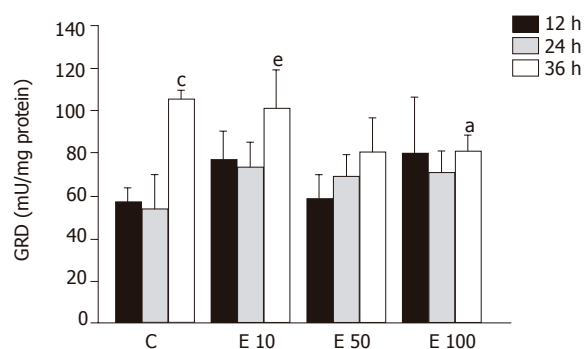


Figure 5 Glutathione reductase (GRD) activities of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a $P < 0.05$ vs 36 h of groups C and E 10; ^c $P < 0.05$ vs 12 h and 24 h of groups C; ^e $P < 0.05$ vs 12 h and 24 h of group E 10.

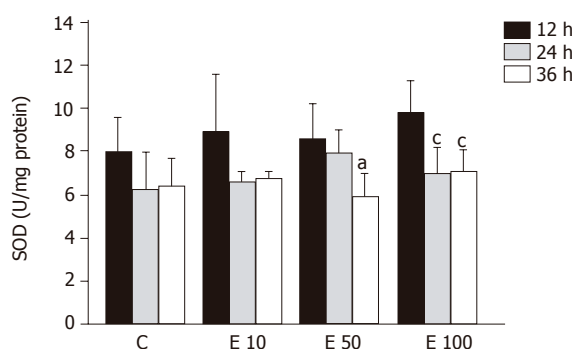


Figure 6 Superoxide dismutase (SOD) activities of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a $P < 0.05$ vs 12 h of group E 50; ^c $P < 0.05$ vs 12 h of group E 100.

After 12-h incubation, CAT activities in groups E50 and E100 were significantly increased, by 94% and 68%, respectively, over those in group C ($P < 0.05$, Figure 7). On the contrary, CAT activity in group E100 at 36 h was significantly decreased from that in groups C and E50 ($P < 0.05$). Moreover, both in groups E50 and E100, CAT activities were significantly lowered at 24 and 36 h from those at 12 h ($P < 0.05$). There was a significant increase in correlation between CAT activity and incubation time ($P = 0.0003$).

DISCUSSION

LDH is an enzyme that exists in many tissues and organs, such as the heart, muscle, kidney, liver, etc. When those tissues or organs are damaged, LDH is released into the blood from the cells. Therefore, LDH leakage can be used to indicate cell viability. In this study, the viability of hepatocytes was monitored by the release of LDH into the medium. That is to say, higher LDH leakage was interpreted as lower viability of hepatocytes. The previous

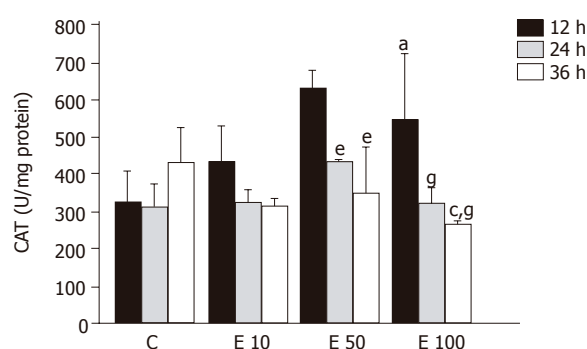


Figure 7 Catalase (CAT) activities of primary rat hepatocytes in each group. Data are mean \pm SD for 3 hepatocyte preparations. Cultures were incubated without ethanol (C), with 10 mmol/L ethanol (E 10), with 50 mmol/L ethanol (E 50), and with 100 mmol/L ethanol (E 100) for 12, 24, and 36 h, respectively. ^a $P < 0.05$ vs 12 h of groups C; ^c $P < 0.05$ vs 36 h of groups C and E 50; ^e $P < 0.05$ vs 12 h of group E 50; ^g $P < 0.05$ vs 12 h of group E 100.

study has shown that ethanol increased the production of ROS and resulted in a decrease in hepatocyte viability^[7]. Results in this study also suggest that high concentration of ethanol (100 mmol/L) may decrease cell viability of hepatocytes.

MDA was one of the main lipid peroxidation products, its elevated levels could reflect the degrees of lipid peroxidation induced injury in hepatocytes^[11]. Many reports have demonstrated that ethanol exposure promotes the accumulation of lipid peroxidation *in vivo*^[12] and *in vitro*^[13]. Lipid peroxidation induced by ethanol administration results from not only increasing ROS production but also the mass generation of acetaldehyde^[14]. Our data also indicated that the MDA concentration showed significant correlation with the incubation concentration and time of ethanol in isolated rat hepatocytes.

It has been reported that chronic ethanol feeding results in increased^[15], decreased^[16], or unchanged hepatic GSH contents^[17] *in vivo*. The discrepancies in the total GSH levels in the livers of rats chronically fed with ethanol might have originated from the differences in the strains of rats used and the dose, duration, and route of ethanol administration among the different studies. On the other hand, the previous study has demonstrated that acute ethanol administration neither decreases nor increases the GSH level in hepatocytes, at least within 4 h after treating with 50 mmol/L ethanol *in vitro*^[18]. Our results also showed that there was no significant difference in GSH content after 50 mmol/L ethanol treatment in hepatocytes. But the GSH concentration in hepatocytes significantly declined after 100 mmol/L ethanol treatment at 24 and 36 h. In this study, the reduction of GSH and the elevation of lipid peroxidation were simultaneously observed in the 100 mmol/L ethanol exposure. Thus, it could be speculated that ethanol-induced lipid peroxidation might contribute to decreased GSH contents in this study as previously shown by others^[18].

Metabolism of ethanol is believed to result in the increased production of ROS, especially superoxide and

H₂O₂, and the removal of these toxic species is thought to be a vital initial step in ensuring cell survival during ethanol intoxication^[6]. Four major antioxidant enzymes available to the cell during ethanol-induced oxidant stress include GPX, GRD, SOD, and CAT. The previous study has shown that GPX, CAT, and SOD activities showed an inverse correlation with the severity of pathological injury in rats fed with ethanol^[12]. In contrast, Oh *et al.* reported that chronic ethanol feeding resulted in the lower activity of GPX with significantly higher activities of GRD and CAT^[15]. However, there have been a few reports about the effects of ethanol on antioxidant enzymes activities in hepatocytes *in vitro*. Our data suggest that higher concentration of ethanol may reduce antioxidant enzymes activities of GRD and CAT after long-time incubation and contribute to decreased cell viability and increased lipid peroxidation.

In conclusion, this study indicates the direct relatedness between ethanol and hepatocytes and excludes the factor of gastrointestinal absorption. It was demonstrated that 100 mmol/L ethanol exposure diminished the cell viability because the lipid peroxidative product accumulated, which was caused by the decreased antioxidative status, including the reduction of GSH contents, GRD and CAT activities.

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Bacterial biota in reflux esophagitis and Barrett's esophagus

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Abstract

AIM: To identify the bacterial flora in conditions such as Barrett's esophagus and reflux esophagitis to determine if they are similar to normal esophageal flora.

METHODS: Using broad-range 16S rDNA PCR, esophageal biopsies were examined from 24 patients [9 with normal esophageal mucosa, 12 with gastroesophageal reflux disease (GERD), and 3 with Barrett's esophagus]. Two separate broad-range PCR reactions were performed for each patient, and the resulting products were cloned. In one patient with Barrett's esophagus, 99 PCR clones were analyzed.

RESULTS: Two separate clones were recovered from each patient (total = 48), representing 24 different species, with 14 species homologous to known bacteria, 5 homologous to unidentified bacteria, and 5 were not homologous (<97% identity) to any known bacterial 16S

rDNA sequences. Seventeen species were found in the reflux esophagitis patients, 5 in the Barrett's esophagus patients, and 10 in normal esophagus patients. Further analysis concentrating on a single biopsy from an individual with Barrett's esophagus revealed the presence of 21 distinct bacterial species. Members of four phyla were represented, including *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. Microscopic examination of each biopsy demonstrated bacteria in intimate association with the distal esophageal epithelium, suggesting that the presence of these bacteria is not transitory.

CONCLUSION: These findings provide evidence for a complex, residential bacterial population in esophageal reflux-related disorders. While much of this biota is present in the normal esophagus, more detailed comparisons may help identify potential disease associations.

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Key words: Bacterial biota; Esophagus; 16S rDNA PCR

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INTRODUCTION

Colonizing bacteria exist in each portion of the human digestive tract, from the oral cavity to the anus. Colonizing bacterial populations are essential for the development of the gastrointestinal mucosal immune system, for the maintenance of a normal physiological environment, and for the provision of essential nutrients^[1,2]. Colonizing bacteria also play a role in a variety of disease conditions, as exemplified by the gastric colonizer *Helicobacter pylori* in relation to gastric cancer^[3]. Conversely, loss of normal biota is responsible for the overgrowth of opportunistic pathogens that normally are inhibited, such as that occurs in antibiotic-associated colitis^[4,5], or in candida vaginitis^[6]. Microenvironment alterations may favor overgrowth of bacteria that produce carcinogenic metabolites^[7-11], promoting tumorigenesis in inflammation-induced cancers, such as adenocarcinoma in experimental colitis mouse models^[12].

A complex bacterial biota has been defined recently in the normal distal esophagus, estimated to be composed of approximately 140 species, of which 95 are identified^[13].

Members of six phyla, *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and TM7 are represented. *Firmicutes* represent the most commonly identified phylum in the distal esophagus, followed by phylum *Bacteroidetes*. Some of the phyla, including *Spirochaetes* and *Deferribacteres* that are commonly represented in the oral cavity, are not identified as esophageal flora, indicating that conditions in the distal esophagus are not ideal for the colonization of all oral flora. These are 14 species identified in the distal esophagus in all four persons studied^[13], indicating that the esophageal biota are unique residents, and not identified simply as organisms transiting from the oral to the gastric cavity. Thus, although the esophagus is generally viewed as a conduit for food passage, the environment in which the bacteria reside is relatively stable.

The distal esophagus may be distinguished from other portions of the esophagus by the changes induced by the reflux of gastric and duodenal contents. Persistent untreated gastroesophageal reflux disease (GERD) can lead to a metaplastic and premalignant condition known as Barrett's esophagus, which carries an increased risk of esophageal adenocarcinoma. Repeated exposure of the distal esophagus to gastric acid and duodenal bile salts likely alters the biota present in the distal esophagus; as has been previously demonstrated in the stomach, changes in the microenvironment lead to alterations in colonizing bacterial populations^[15]. One possibility is that perturbation of the normal esophageal biota could contribute to the progression from GERD to Barrett's esophagus towards the development of adenocarcinoma. Because little is known about the nature of bacterial biota in reflux esophagitis-related diseases, we sought to identify whether there exists a population of bacteria in patients with GERD and Barrett's esophagus. Our specific goal in this pilot study was to use broad-range 16S rDNA PCR to identify the presence of colonizing bacteria in patients with reflux esophagitis or Barrett's esophagus.

MATERIALS AND METHODS

Subjects

Patients presenting to the Department of Veterans Affairs Medical Center, Nashville, TN, USA with gastrointestinal symptoms requiring upper gastrointestinal endoscopy were eligible for this study. Those who were willing to participate in the studies of upper gastrointestinal microbiology and who signed an informed consent form were recruited for this study^[16]. Exclusion criteria included recent use of antibiotics, previous gastric/esophageal surgery, and active infection of the oral cavity^[17]. Esophagogastroduodenoscopy was performed and endoscopic findings were recorded for 24 consecutive patients who met the above criteria. Esophageal biopsies were obtained 2 cm above the squamocolumnar junction or in the case of Barrett's esophagus, 2 cm above the gastroesophageal junction. Each biopsy was examined microscopically for morphological features of GERD and

intestinal metaplasia (Barrett's esophagus). As described, features consistent with GERD included mucosal erosions/superficial ulcerations, epithelial hyperplasia, and inflammatory infiltrate of polymorphonuclear cells or eosinophils in the mucosal layer. Features of Barrett's esophagus included the presence of intestinal-type epithelium in the esophagus^[18]. Tissue sections of esophageal biopsies from representative patients with normal esophagus, esophagitis, or Barrett's esophagus were examined by microscopy using Gram-Twort stain^[19].

Specimen processing for molecular biological studies

Biopsies of 2 mm×2 mm×2 mm obtained for this study were placed in a 1.5-mL screw-top test tube and stored at -70 °C. The specimens were coded so that the laboratorian performing the studies was blinded to the clinical information. DNA was extracted from the biopsy using a tissue DNA extraction kit (Qiagen) in a PCR-free clean-room and the DNA-enriched fractions were eluted in 200 microliters of buffer, as described by the manufacturer.

PCR

For each PCR amplification, 5 microliters of the DNA extracted from each biopsy was added to 45 µL of PCR reaction mixture containing 5 µL of 10× PCR buffer (Qiagen), 1.5 mmol/L MgCl₂, 200 µmol/L each dNTP, 50 pmol of each primer, and 5 units of Taq DNA polymerase. Reactions were run at 94 °C for 2 min, followed by 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a 10-min extension at 72 °C. Primers used were fPB7I (forward): 5'-GGIACTGAGACACIGICCCIIACTCCT-3' and rPB10I (reverse): 5'-CGTATTACCGCIGCTGCTGGCAC-3', where I represents inosine, which was used at positions of nucleotide ambiguity, since it forms stable base pairs with A, G, T, and C. Use of inosine-containing primers significantly reduces the complexity accompanying the use of conventional degenerate primers^[20-22]. As such, both inosine-containing primers perfectly match the consensus sequence-derived 16S rDNA pools composed of 21 evolutionarily-well diversified eubacterial groups including *Agrobacterium*, *Aquifex*, *Arthrobacter*, *Bacillus*, *Chlamydia*, *Chlorobium*, *Chloroflexus*, *Chloroplast*, *Clostridium*, *Desulfovibrio*, *Escherichia*, *Flavobacterium*, *Flexibacter*, *Gloeobacter*, *Hellobacterium*, *Leptonema*, *Planctomyces*, *Rhodocyclus*, *Synechococcus*, *Thermotoga*, and *Thermus*^[23], but do not have significant 3' homology with human 18S rDNA, and human mitochondrial small subunit rDNA sequences. The expected PCR products are approximately 210 bp, depending on the species. In a study to determine the sensitivity, the above primer pair was able to amplify as little as one copy of an *Escherichia coli* genome (data not shown). An amplification control was designed to assess whether DNA extracted from the esophageal biopsy is of sufficient quality and quantity to be amplified by PCR. A primer pair specific for human 18S rDNA

was designed to serve this purpose: PBH (forward), 5'-TTGCCAAGAAATGTTTTC-3' and rPBH (reverse), 5'-CGCGTAACTAGTTAGCA-3'.

Cloning and sequencing

The PCR products were separated from free PCR primers using a PCR purification kit (Qiagen), then ligated with the pGEM® T Easy (Promega) vector, and used to transform into *E. coli* DH5 α competent cells. The cloned inserts underwent sequence analysis using vector-based primers.

Phylogenetic analysis

Primer sequences were removed from all sequence files, and only inter-primer sequences were used in subsequent analyses. The sequences were analyzed using standard nucleotide BLAST (Blastn) search of GenBank for homology with known bacterial 16S rDNA sequences. In this study, 16S rDNA sequences with >97% identity with known sequences were considered as homologous with known bacterial species, as described^[24]. 16S rDNA sequences were aligned using ClustalW^[25] and phylograms of nucleotide alignments generated using Paup 4.0b10 (Paup 4.0b2. Phylogenetic Analysis Using Parsimony and Other Methods, Version 4, Sinauer Associates, Sunderland, MA, USA) neighbor-joining method based on HKY85 distance matrices^[26]. All novel sequences were deposited in GenBank (accession numbers: AY212255-21225564).

RESULTS

Microscopic examination of bacterial flora in the distal esophagus

The 24 patients examined included 9 with normal esophagus, 12 with esophagitis, and 3 with Barrett's esophagus (Table 1). Because chronic gastritis is generally associated with the presence of overlying *H. pylori* in the lumen^[27], we examined the inflamed distal esophagus to determine whether bacterial cells might be visible. Such a study, if positive, can provide morphological evidence for an indigenous esophageal biota, and provide a rationale for its further characterization. Of the 24 biopsies, 21 had sufficient tissue materials remaining for Gram-Twort stain, including 6 from patients with normal esophagus, 12 with esophagitis, and 3 with Barrett's esophagus. Bacteria were observed in 52% of the biopsies (from 4 of 6 with normal esophagus, 5 of 12 with esophagitis, and 2 of 3 with Barrett's esophagus). Bacteria appeared to be closely associated with the epithelial cell surfaces (Figure 1). All bacteria observed in the two biopsies with Barrett's esophagus were Gram-positive cocci, while in the 9 non-Barrett's biopsies, all were Gram-negative cocci or bacilli.

Bacterial 16S rDNA in esophageal biopsy specimens

To examine the nature of the bacterial populations present in the distal esophagus and to define their ancestry, we performed universal bacterial 16S PCR on biopsies

Table 1 Clinical and pathological features of 24 patients included in this study¹

Case#	Age ²	Symptoms of heartburn		Esophageal features	
		Frequency (time/week)	Duration ³	Endoscopic diagnosis	Histopathology ⁴
Normal esophagus (<i>n</i> = 9)					
248	73	0	NA	Normal	NP
251	66	0	NA	Normal	NP
256	66	0	NA	Normal	NP
259	75	3	1 Y	Hiatal hernia	NP
261	81	3	>1 Y	Esophageal ring	NP
262	65	0	NA	Esophagitis	NP
263	51	3	1 Y	Gastric erosions	NP
264	72	0	NA	Esophageal ring	NP
265	71	0	NA	Hiatal hernia	NP
Esophagitis (<i>n</i> = 12)					
247	63	3	>1 Y	Esophagitis	AI, CI, EO
252	51	0	NA	Normal	AI, CI, EO
254	57	3	1 Y	Esophageal ring	AI, CI, EO
257	52	3	1 M	Esophagitis	AI, CI, EO
249	34	0	NA	Esophagitis	AI, CI
243	53	3	>1 Y	Esophagitis	CI, EO
255	66	3	>1 Y	Esophagitis	CI, EO
258	52	3	2-3 Y	Barrett's esophagus	CI, EO
244	74	3	2 M	Esophagitis	CI
250	68	0	NA	Esophageal ring	CI
260	75	3	2-3 M	Esophagitis	CI
253	71	5	4 Y	Esophagitis	CI
Barrett's esophagus (<i>n</i> = 3)					
242	69	0	NA	BE	BE
245	70	0	NA	BE	BE
246	66	3	>1 Y	BE	BE, CI, EO

¹None of the 24 patients had a history of recent use of antibiotics, previous gastric/esophageal surgery, active infection of the oral cavity^[17].

²All 24 patients are males, reflecting the veterans population.

³NA, not available; Y, year; M, month.

⁴NP: no pathological changes; BE: Barrett's esophagus; EO: eosinophils; CI: chronic inflammation; AI: acute inflammation.

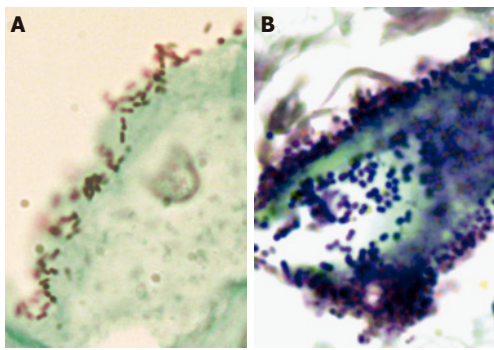


Figure 1 Microscopic examination of bacterial cells in the esophagus. Esophageal biopsies were fixed in formalin, paraffin-embedded, sectioned, and examined by using Gram-Twort stain. **A:** In the biopsy from patient #265 with a normal esophagus, Gram-negative cocci and coccobacilli were tightly associated with the surface of squamous epithelial cells. **B:** In the biopsy from patient #246 with Barrett's esophagus, Gram-positive cocci were highly concentrated within the lumen of an intestinal-type gland.

from each of the 24 studied patients. From each of the 24 biopsies, 2 clones of PCR products were randomly picked and sequenced. The 48 samples yielded 36 unique sequences belonging to 24 different species (97% identical), as established through GenBank BLAST searches. Of the 24 species identified, 14 represented known cultivation-defined bacterial species by sharing 97% identity, 5 shared 97% identity to 5 noncultured/unidentified bacterial species, and 5 did not share significant homology (<97% identity) with any existing bacterial 16S rDNA sequences in the GenBank (Table 2).

Of the 48 clones sampled, unidentified oral bacterium SH66 was the most prevalent species amplified, accounting for 22.9% (11 clones from 9 patients), followed by *Prevotella veroralis* (10.4%, 5 clones from 5 patients), members of the *Streptococcus* genus (10.4%, 5 clones from 5 patients), and *H. pylori* (6.3%, 3 clones from 2 patients) (Table 2). None of the remaining species constituted more than 5% of

Table 2 Analysis of 48 bacterial 16S rDNA sequences detected in the esophageal biopsies from 24 patients

Best matched bacterial 16S rDNA	% Identity ¹	Number of sequences detected			
		Normal esophagus (n = 9) ²	Esophageal disease		All patients (n = 24)
			Esophagitis (n = 12)	Barrett's (n = 3)	
Cultivation-defined species ³ (n = 14)					
<i>Prevotella veroralis</i>	98.2	4	1		5
<i>Streptococcal species</i>	100	1	2		3
<i>Pseudomonas species</i>	100		2	1	3
<i>Helicobacter pylori</i>	100, 99.3, 98.6		3		3
<i>Prevotella pallens</i>	100	1		1	2
<i>Streptococcus salivarius</i>	100, 99.4	1		1	2
<i>Actinobacillus pleuropneumoniae</i>	99.4, 98.8	1	1		2
<i>Acinetobacter</i> sp. OM-E81	100			1	1
<i>Citrobacter amalonaticus</i>	100		1		1
<i>Haemophilus influenzae</i>	100		1		1
<i>Haemophilus parainfluenzae</i>	99.4		1		1
<i>Veillonella atypica</i>	99.4	1			1
<i>Campylobacter fetus</i>	97.9	1			1
<i>Prevotella oulora</i>	97		1		1
Subtotal		10	13	4	27
Unidentified species ⁴ (n = 5)					
Oral bacterium SH66	100, 99.4, 98.8	6	3	2	11
Oral bacterium RP55-18	100, 99.4		2		2
Oral bacterium SH13	100	1			1
Oral bacterium SH64	99.4	1			1
Oral bacterium AP60-12	98.3		1		1
Subtotal		8	6	2	16
Unknown ⁵ (n = 5)					
(Bacterium CEC2)	96.5		1		1
(<i>Veillonella ratti</i>)	93		1		1
(<i>Cytophagales</i>)	92.1		1		1
(Marine bacterium SS1)	89.9		1		1
(Rumen bacterium RFN91)	89.2		1		1
Subtotal			5		5

¹Multiple numbers indicate that sequences were obtained from more than one clone, and that the identity to the specified best matching bacteria varied between clones.

²n indicates number of patients in each group.

³Cultivation-defined species: 16S rDNA sequence with equal or greater than 97% identity with 16S rDNA of cultivation-defined bacterial species^[24].

⁴Unidentified species: 16S rDNA sequence with equal or greater than 97% identity with PCR-derived 16S rDNA sequence^[24].

⁵Unknown: 16S rDNA sequence with <97% identity with any known 16S rDNA sequences.

the sequenced pool. Cultivation-defined and unidentified species were distributed to nearly the same extent between specimens from normal or diseased esophagus. Of the 27 clones of cultivation-defined bacterial species, 10 were from patients with normal esophagus and 17 were from the 15 patients with esophageal diseases. Of the 16 clones of unidentified/noncultured species, 8 were from

the 15 patients with normal esophagus and 8 were from the 9 patients with esophageal disease. In contrast, the 5 clones of novel species were all from the 15 patients with esophageal diseases. In total, 17 species were found in reflux esophagitis, 5 in Barrett's esophagus, and 10 in the normal esophagus.

Colonization of the bacterial populations within a single esophageal biopsy

To characterize the bacterial populations within a single esophageal biopsy in the presence of esophageal disease, we further analyzed the biota from a patient with Barrett's esophagus (case 242; Table 3). From this biopsy, 99 clones were randomly picked and sequenced to allow a more in-depth analysis of the bacterial population in this biopsy. The 99 clones contained 36 unique 16S rDNA sequences comprising 21 species, including 10 homologous to cultivation-defined bacterial species, 5 homologous to unidentified/noncultured species, and 6 without significant homology (<97% identity) to any known 16S sequences at the species level (Table 4). Unidentified oral bacterium SH66 represented the most prevalent bacterial species (50.5% of the clones sequenced), followed by *Neisseria flavescens* (11.1%) and *Prevotella pallens* (6%).

Through combining the preliminary examination of the 24 patients, and the in-depth examination of a single patient with Barrett's esophagus, 147 sequences were obtained, belonging to 39 different species (Figure 2). Twenty-two of the sequences were homologous with cultivation-defined bacterial species, 7 with uncultivated species, and 10 were not homologous with any known bacterial species. The clones belonged to four phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (Figure 2).

DISCUSSION

Although 147 sequences from the biopsy specimens were analyzed, this study must be considered preliminary. Our strategy was to sample small populations of patients with reflux-related esophageal diseases to determine whether bacterial biota exist and ascertain any outstanding

Table 3 Prevalence of specific 16S rDNA in 99 subclones from a single biopsy from patient #242 with Barrett's esophagus

Best matched bacterial 16S rDNA	% Identity ¹	Number of sequences
Cultivation-defined species ² (n = 10)		
<i>Neisseria flavescens</i>	98.8, 98.2	11
<i>Prevotella pallens</i>	100	6
<i>Porphyromonas</i> sp oral clone CW034	100, 99.4, 97.6	3
<i>Gemella morbillorum</i>	100	2
<i>Prevotella</i> sp oral clone BI027	100	1
<i>Campylobacter fetus</i>	99.4	1
<i>Rothia mucilaginosa</i>	99.4	1
<i>Veillonella</i> sp oral clone AA050	98.8	1
<i>Veillonella parvula</i>	98.2	1
<i>Catonella morbi</i>	98.1	1
Subtotal		28
Unidentified species ³ (n = 5)		
Oral bacterium SH66	100, 99.4	50
Oral bacterium SH25	99.4	4
Oral bacterium SH13	100	1
Oral bacterium AP60-12	98.7	1
Oral bacterium AP60-35	98.2, 97.6	2
Subtotal		58
Unknown ⁴ (n = 7)		
(<i>Prevotella</i> sp oral clone FO45)	95	3
(Rumen bacterium JW17)	93.9	3
(Rumen bacterium RFN91)	88.4, 89	3
(Rumen bacterium 30-15)	94.5, 93.9	2
(Rumen bacterium JW17)	96.3	1
(<i>Prevotella</i> sp oral clone AH125)	93.9	1
Subtotal		13

¹Multiple numbers indicate that sequences were obtained from more than one clone, and that the identity to the specified best matching bacteria varied between clones.

²Cultivation-defined species: 16S rDNA sequence with equal or greater than 97% identity with 16S rDNA of cultivation-defined bacterial species^[24].

³Unidentified species: 16S rDNA sequence with equal or greater than 97% identity with PCR-derived 16S rDNA sequence^[24].

⁴Unknown: 16S rDNA sequence with <97% identity with any known 16S rDNA sequences.

Table 4 Comparison of representation of bacterial phyla observed in studies of the esophagus and subgingival crevice

Phylum	Subgingival crevice			Esophagus			
	Number of clones		Number of species	Number of clones		Number of species	
	Paster ¹	Paster ¹		Pei ³	Present study	Present study	Present study
Clostridium group	0	0	13	0	0	0	0
Obsidian pool OB11	6	1	0	0	0	0	0
Deferribacteres	86	8	8	0	0	0	0
Spirochaetes	537	58	0	0	0	0	0
TM7	34	5	0	13	3	0	0
Fusobacteria	353	19	2	20	6	0	0
Actinobacteria	275	32	15	39	8	2	2
Firmicutes	659	113	15	626	41	18	11
Proteobacteria	338	51	14	20	14	31	11
Bacteroidetes	234	58	8	182	23	96	15
Total	2522	347	75	900	95	147	39

¹From Paster *et al.*^[14]; ²from Kroes *et al.*^[28]; ³from Pei *et al.*^[13].

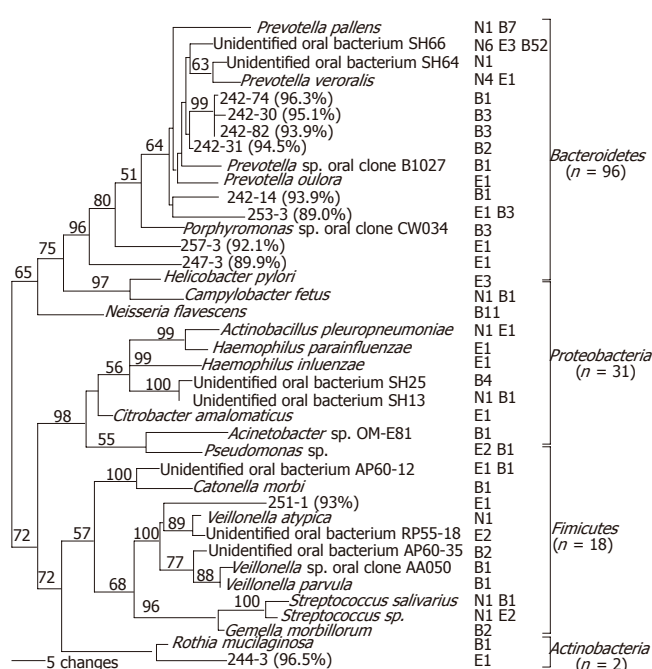


Figure 2 Phylogenetic analysis of bacterial 16S rDNA detected in the distal esophagus. Sequences were aligned using ClustalW, and subjected to phylogenetic analysis using Paup 4.0b10 neighbor-joining analysis, based on HKY85 distance matrices. Bootstrap values (based on 500 replicates) are represented at each node when >50%, and the branch length index is represented below the phylogram. Names of bacterial species closest in homology with the detected 16S rDNA are located at the termination of each branch. "Unidentified" oral bacterial clones are potential bacterial species whose phylogenetic positions were designated by PCR-amplified 16S sequences only, rather than based on cultured organisms. The serial number of a clone followed by percentage of homology with the closest DNA sequence in GenBank is used as the species name for 16S rDNA sequence with <97% identity with all known DNA sequences. The frequency that a species was detected and its sources are shown following the species name. 'N' represents normal esophagus, 'E' represents esophagitis, and 'B' represents Barrett's esophagus. The 39 species belong to four phyla, as shown at the right.

associations. The aim of these studies was thus hypothesis-generating, to establish parameters for more definitive studies. This approach was necessary because when this study was begun there was no prior information relating to bacterial populations in reflux-related disorders. We recognize the preliminary nature of this inquiry, but believe it can serve as a first approximation that can help guide future work.

Our findings suggest the existence of highly complex bacterial populations in the distal esophagus of patients with GERD-related disorders. Because many bacteria are fastidious, slow growing, or even uncultivable, simple culture methods often overlook a large number of bacteria, such as has been documented in the oral cavity and colon^[14,28-30]. These drawbacks can be overcome by universal bacterial 16S rDNA PCR, since PCR does not discriminate bacteria based on their culture properties. However, PCR cannot distinguish living bacteria from naked bacterial genomes. In organs in which major digestive activities occur, such as the stomach and small intestine, bacteria brought downstream by peristaltic movement may be lysed and genomes released. Such

DNA can be falsely interpreted by PCR as representing colonizing bacteria. Using microscopy in the present study, we observed a polymorphic population of bacteria in association with the epithelium of esophageal biopsies from patients with reflux-related diseases that included Gram-positive and Gram-negative bacilli, cocci, and coccobacilli. The variety of morphologically diversified esophageal bacteria is consistent with the highly diversified bacterial constituents identified using molecular techniques. The presence of intact bacteria closely associated with epithelial cells of the distal esophagus suggests that the 16S rDNA detected was from viable bacteria rather than from bacterial DNA only.

Because only a few PCR clones were sampled from each biopsy specimen, this study is not a quantitative comparison of the bacteria found in GERD-related disorders with those from normal esophagus or oral cavity^[13,14,28-30], as the species identified may reflect chance rather than prevalence. However, the majority of bacterial species found in GERD-related disorders are shared with the previously identified bacteria in the normal esophagus^[13], suggesting that certain bacterial species in the normal esophagus are resistant to the substantial environmental changes due to reflux. Finding *H. pylori* 16S rDNA in the esophagus of two patients with GERD indicates that gastric bacteria can be brought into the distal esophagus by reflux, consistent with the previous detection of *H. pylori* in Barrett's esophagus^[31,32]. These observations suggest that the detected organisms (or DNA) may be transiently present, rather than persistent in the distal esophagus. Longitudinal studies of individual patients would help to address this question. Conversely, the microenvironment in the distal esophagus likely does not include all oral bacteria. Similar to our previous study of the normal esophageal biota^[13], the most prevalent of the nine phyla identified in the subgingival crevice^[14,28], *Spirochaetes*, was not found in the esophagus, consistent with the presence of endogenous bacterial populations unique to the distal esophagus.

In-depth study of a Barrett's esophagus case (Biopsy 242) revealed a single predominant species (unidentified oral bacterium SH66), representing 50.5% of the 99 clones sampled. SH66 was found in patients with or without the disease of the distal esophagus; whether there is overgrowth in Barrett's esophagus cannot be addressed without quantitative comparisons. SH66 was originally identified in the saliva by its 16S sequence, but never has been cultured^[33]. Phylogenetic analysis indicates that SH66 resembles several members of the genus *Prevotella* and belongs in the phylum *Bacteroidetes* (Figure 2).

Identifying complex bacterial populations in GERD-related disorders offers a new approach to understand bacterial roles as markers or as pathogenic factors in esophageal diseases. Bacterial populations in other portions of the digestive system, such as the oral cavity and colon, play important roles in the maintenance of local physiology as well as in disease pathogenesis^[1-5,6-10,11-13]. The composition, transience, or stability of this complex bacterial biota in the distal esophagus and associations

with the disease remain to be determined. The results from this study justify large-scale comparisons of bacterial biota between normal and pathological conditions in the distal esophagus.

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• CLINICAL RESEARCH •

Lymphomatous involvement of gastrointestinal tract: Evaluation by positron emission tomography with ^{18}F -fluorodeoxyglucose

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Abstract

AIM: To demonstrate the ^{18}F -fluorodeoxyglucose positron emission tomography (^{18}F -FDG PET) findings in patients with non-Hodgkin's lymphoma (NHL) involving the gastrointestinal (GI) tract and the clinical utility of modality despite of the known normal uptake of FDG in the GI tract.

METHODS: Thirty-three patients with biopsy-proven gastrointestinal NHL who had undergone FDG-PET scan were included. All the patients were injected with 10-15 mCi FDG and scanned approximately 60 min later with a CTI/Siemens HR (+) PET scanner. PET scans were reviewed and the maximum standard uptake value (SUV_{max}) of the lesions was measured before and after the treatment, if data were available and compared with histologic diagnoses.

RESULTS: Twenty-five patients had a high-grade lymphoma and eight had a low-grade lymphoma. The stomach was the most common site of the involvement (20 patients). In high-grade lymphoma, PET showed focal nodular or diffuse hypermetabolic activity. The average $\text{SUV}_{\text{max}} \pm \text{SD}$ was 11.58 ± 5.83 . After the therapy, the patients whose biopsies showed no evidence of lymphoma had a lower uptake without focal lesions. The $\text{SUV}_{\text{max}} \pm \text{SD}$ decreased from 11.58 ± 5.83 to 2.21 ± 0.78 . In patients whose post-treatment biopsies showed lymphoma, the $\text{SUV}_{\text{max}} \pm \text{SD}$ was 9.42 ± 6.27 . Low-grade follicular lymphomas of the colon and stomach showed diffuse hypermetabolic activity in the bowel wall (SUV_{max} 8.2 and 10.3, respectively). The SUV_{max} was 2.02-3.8 (mean 3.02) in the stomach lesions of patients with MALT lymphoma.

CONCLUSION: ^{18}F -FDG PET contributes to the diagnosis of high-grade gastrointestinal non-Hodgkin's lymphoma,

even when there is the normal background FDG activity. Furthermore, the SUV plays a role in evaluating treatment response. Low-grade NHL demonstrates FDG uptake but at a lesser intensity than seen in high-grade NHL.

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Key words: Positron emission tomography; Non-Hodgkin's lymphoma; Gastrointestinal neoplasm

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INTRODUCTION

Non-Hodgkin's lymphoma (NHL) is known to arise from extranodal sites in 10-30% of cases^[1,2]. Among the extranodal sites, the gastrointestinal tract is most frequently affected by NHL. It can involve the gastrointestinal tract partly or entirely^[3]. Generally, the diagnosis of lymphomatous involvement of the gastrointestinal tract is based on clinical symptoms and imaging studies, such as double-contrast barium study, computed tomography (CT) and is confirmed by endoscopy with biopsy. CT has been widely used as the imaging modality for staging and restaging of lymphoma^[4]. However, in evaluating lymphomatous involvement of the gastrointestinal tract, CT has some limitations. Examples are non-specific imaging patterns and findings that may be difficult to interpret such as wall thickening in a non-distended stomach or unopacified small bowel loops^[5-7]. Gallium-67 citrate scintigraphy also plays an important role in patients with NHL for the detection of lesions, initial staging and assessment of therapeutic responses^[8,9]. Gallium-67 citrate scintigraphy is known to be more sensitive for the detection of lesions in thoracic locations, but it is much less sensitive in the identification of infradiaphragmatic sites owing to physiologic hepatic and splenic uptake and excretion into the bowel^[10]. Since Paul^[11] first reported ^{18}F -FDG PET imaging in lymphoma, this modality has been increasingly used to examine patients with lymphomas. Many published articles have shown that ^{18}F -FDG PET is more sensitive in detect-

ing disease sites than gallium-67 scintigraphy. ^{18}F -FDG PET is at least as sensitive as CT, but more specific than CT, especially in patients undergoing restaging^[12-15]. In addition, many studies have demonstrated that persistent FDG uptake after therapy may predict treatment failure or a high recurrence rate^[16,17]. Most reported studies have assessed primarily patients with nodal NHL. A few studies have used ^{18}F -FDG PET to assess NHL in the gastrointestinal tract^[18-21]. Rodriguez *et al.*^[18] demonstrated that ^{18}F -FDG PET may have a novel application in the evaluation of gastric NHL and may complement endoscopy and CT in selected patients. However, in a study by Hoffmann *et al.*^[19] in patients with mucosa-associated lymphoid tissue (MALT)-type lymphoma, no focal tracer uptake was demonstrated with ^{18}F -FDG PET in either gastric or extragastric lesions. In addition, there is concern that normal FDG accumulation in the gastrointestinal tract or abnormal uptake in patients with inflammatory bowel disease could cause confusion in the interpretation of ^{18}F -FDG PET images in patients with lymphoma^[22-25]. Thus the clinical utility of ^{18}F -FDG PET imaging in the evaluation of lymphomatous involvement of the gastrointestinal tract has not been clearly established. This study aimed to demonstrate the ^{18}F -FDG PET findings in patients with NHL (high-grade *vs* low-grade) involving the gastrointestinal tract and the clinical utility of this modality through the normal uptake of FDG in the gastrointestinal tract was known.

MATERIALS AND METHODS

In this study, the electronic database of 907 consecutive patients with lymphoma who underwent PET imaging from October 2000 to June 2002 was retrospectively reviewed. Thirty-three patients with biopsy-proven NHL involving the gastrointestinal tract who underwent a ^{18}F -FDG PET scan were included in this study. There were 24 men and 9 women aged 34-80 years (mean 58 years). For scanning, all patients were injected with 10-15 mCi FDG and scanned approximately 60 min later with a CTI/Siemens HR (+) PET scanner (Siemens, Knoxville, TN, USA). Each scan was performed from the head to the pelvic floor with the total time of about 60 min for image acquisition. The acquired data were reconstructed using standard vendor-provided iterative reconstruction with segmented attenuation correction. Additional transmission scanning for attenuation correction was performed.

The PET scans obtained were reviewed by an experienced nuclear physician and a radiologist who together provided the consensus reading. Reviewing was done without patient's clinical data and the status of lymphoma. The SUV_{max} of the lesions was measured before and after the treatment in case the data were available. The SUV_{max} was measured in at least two orthogonal planes to demonstrate the best lesion appreciation. The CT scan obtained on the corresponding data was used as the guideline for demarcating those lesions when their boundaries were difficult to define. The highest SUV_{max} was used for each lesion. The ^{18}F -FDG

Table 1 Sites of gastrointestinal involvement of NHL in 33 patients

Sites of GI involvement	Number of patients (%)
Stomach	21 (64)
Colon and rectum	12 (36)
Terminal ileum and cecum	9 (27)
Duodenum	8 (24)
Small bowel	6 (18)
Esophagus	2 (6)

PET results and the SUV measurements at each time point were compared with the histologic data from the corresponding data. The SUV_{max} was compared before and after the treatment using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Of the 33 patients with NHL involving the gastrointestinal tract, 25 had a high-grade lymphoma and 8 had a low-grade lymphoma as determined using the revised European-American classification of lymphoid neoplasm (REAL classification). The histologic subtypes included diffuse large cell lymphoma ($n = 16$), mantle cell lymphoma ($n = 6$), MALT-type lymphoma ($n = 5$), peripheral T-cell lymphoma ($n = 3$), follicular lymphoma ($n = 2$) and B-cell small lymphocytic lymphoma ($n = 1$). The stomach was the most common site of the involvement, followed (in order of decreasing prevalence) by the colon and rectum, cecum and terminal ileum, duodenum, small bowel, and esophagus (Table 1).

In high-grade NHL, ^{18}F -FDG PET showed focal nodular or diffuse hypermetabolic activity, which involved mainly the wall of the gastrointestinal tract. This activity's appearance was different from that of the normal activity in the bowel wall, which was less intense and uniform. Fifteen patients received ^{18}F -FDG PET for staging before the treatment. In these patients, ^{18}F -FDG PET identified 30 (100%) of 30 intestinal locations that had biopsy-proven lymphomatous involvement. These locations were the esophagus ($n = 2$), stomach ($n = 11$), duodenum ($n = 5$), small bowel ($n = 6$), cecum/terminal ileum ($n = 3$), and colon ($n = 3$). The SUV_{max} ranged from 3.64 to 25.10 (11.22 ± 5.79). The $\text{SUV}_{\text{max}} \pm \text{SD}$ in non-involved intestine was 3.09 ± 2.34 . After the therapy, uptake was absent or reduced without focal lesions in 14 patients who had complete responses and whose biopsies showed benign inflammation without evidence of lymphoma (Figure 1). The post-treatment SUV_{max} values were significantly lower ($P < 0.05$), ranging from 0.89 to 4.30 (2.21 ± 0.78). In three patients, the post-treatment biopsies still showed lymphoma (Figure 2) and the post-treatment SUV_{max} ranged from 3.44 to 21.90 (9.42 ± 6.27).

In low-grade NHL, ^{18}F -FDG PET showed diffuse hypermetabolic activity in the bowel wall in two patients with follicular lymphoma of the colon and stomach (SUV_{max} 8.20 and 10.30, respectively). In one patient with follicular lymphoma of the stomach, the SUV_{max} after 5 wk of therapy decreased from 10.30 to 2.40. In four patients with

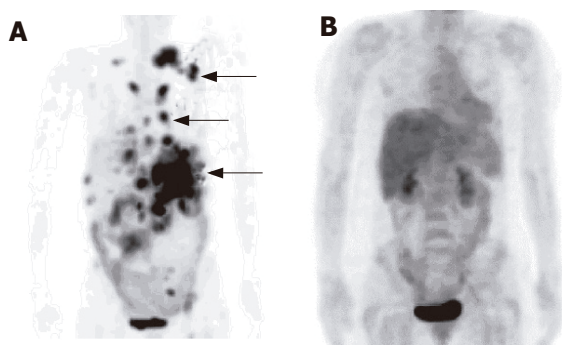


Figure 1 A 61-year-old woman with large cell lymphoma. **A:** Pre-treatment ^{18}F -FDG PET scan revealed multifocal hypermetabolic activity involving the neck, chest, and abdomen (arrow). The highest SUV_{max} was 20.3 in the left upper abdominal area, corresponding to a positive result of a gastric biopsy; **B:** Follow-up ^{18}F -FDG PET scan 5 mo later showed no evidence of residual FDG disease.

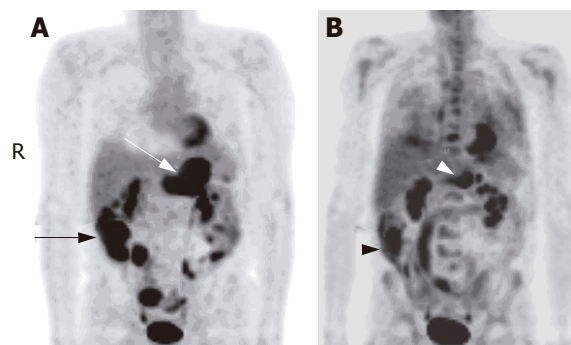


Figure 2 A 68-year-old man with large cell lymphoma. **A:** Base line ^{18}F -FDG PET scan showed hypermetabolic foci in the stomach (white arrow), cecum and terminal ileum (black arrow), and bowel loops ($\text{SUV}_{\text{max}} = 12.7$); **B:** ^{18}F -FDG PET scan obtained after therapy showed partial metabolic response of the activity in the stomach (white arrowhead) and cecum (black arrowhead, $\text{SUV}_{\text{max}} = 10.7$).

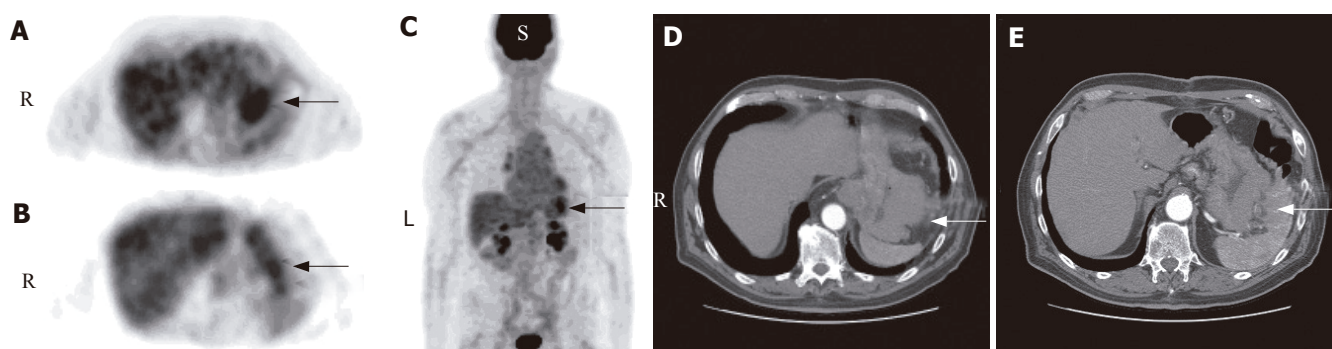


Figure 3 An 80-year-old man with MALT-type lymphoma. **A-C:** ^{18}F -FDG PET scans in transaxial and projection images showed a focal mild hypermetabolic activity ($\text{SUV}_{\text{max}} = 3.8$) in the region of stomach (black arrow); **D and E:** CT scans showed a bulky mass in the wall of gastric fundus and body (white arrow).

MALT-type lymphoma (Figure 3), lesions in the stomach and duodenum showed diffuse and low metabolic activity similar to that in the liver, and the SUV_{max} was 2.02-3.8 (average 3.02) at the time of the positive biopsy results. In another patient with MALT-type lymphoma involving the colon, the SUV_{max} was 6.82. However, follow-up biopsy in this patient showed high-grade transformation. The mean SUV_{max} in high-grade and low-grade NHL before and after the treatment is shown in Table 2. Negative post-treatment biopsy results corresponded to significantly decreased SUV_{max} in high-grade NHL ($P < 0.05$). However, the SUV_{max} before and after the treatment was not significantly lower in low-grade NHL.

DISCUSSION

The gastrointestinal tract is the most common extranodal site of NHL^[1,3]. The stomach is most frequently involved (60-74% of cases), followed by the duodenum and small bowel (10-20%), ileocecal region (7-10%) and large bowel (<10%)^[26,27]. Our results are consistent with these reports, except that the large bowel was involved in a greater percentage of patients in our study than the previous studies. Lymphomatous involvement of the gastrointestinal tract may be primary or secondary. The gastrointestinal tract is involved at autopsy in as many as

Table 2 SUV_{max} in high- and low-grade NHL before and after treatment (mean \pm SD)

NHL grade	SUV_{max} before treatment	SUV_{max} after treatment	
		Biopsy positive	Biopsy negative
High grade	11.22 \pm 5.79 (n = 15)	9.42 \pm 6.27 (n = 14)	2.21 \pm 0.78 ^{a,c} (n = 3)
Low grade	8.57 \pm 2.47 (n = 2)	3.76 \pm 2.08 (n = 5)	3.03 \pm 0.89 (n = 2)

n, number of patients.

^a $P < 0.05$ vs SUV_{max} in high grade NHL before treatment; ^c $P < 0.05$ vs SUV_{max} with biopsy positive after treatment.

50% of patients with secondary lymphoma. However, most of these patients have subclinical disease while patients who have gastrointestinal symptoms are found to have primary gastrointestinal lymphoma^[6,28].

Diagnostic imaging studies play an important role in documenting lymphoma, staging and re-staging the disease, evaluating treatment response and performing follow-up evaluations. Anatomical imaging modalities including computed tomography (CT) and magnetic resonance (MR) imaging have some limitations, especially when defining the viability of the residual mass, treatment

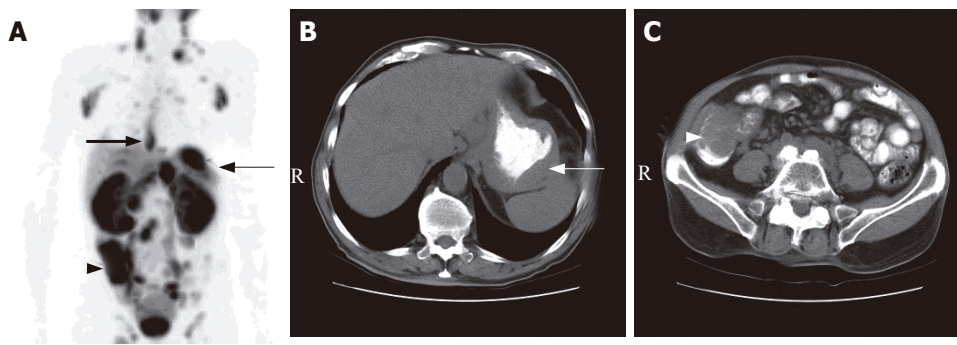


Figure 4 A 78-year-old man with mantle cell lymphoma. **A:** ^{18}F -FDG PET scan revealed multiple hypermetabolic foci involving the lower esophagus (thick arrow), gastric fundus (thin arrow), cecum and terminal ileum (arrow head); **B** and **C:** The corresponding CT scans of the abdomen showed wall thickening at the gastric fundus (white arrow) and the cecum and terminal ileum (white arrow head)

response or both^[4,29]. Gallium-67 scintigraphy has been proposed as a functional imaging modality to assess remission and to evaluate the nature of residual masses in patients with lymphoma. However, gallium-67 scintigraphy is of little use in the abdomen because of the high hepatic uptake and excretion into the bowel and should be performed before treatment to determine whether the patient has a gallium-fixing tumor and whether the absence of fixation after treatment corresponds to a residual mass^[8,9,30]. ^{18}F -FDG PET imaging has shown its clinical usefulness in patients with lymphoma. Several articles have reported that ^{18}F -FDG PET has a higher sensitivity in detecting disease sites than gallium-67 scintigraphy^[11-13]. ^{18}F -FDG PET can also be used to assess the response after the therapy^[16,17,31]. However, a limited number of studies have shown the utility of ^{18}F -FDG PET imaging for the evaluation of lymphomatous involvement of the gastrointestinal tract^[18-21,32,33].

In our study, ^{18}F -FDG PET showed fixed focal nodular or diffuse hypermetabolic activity of all lesions in patients with high-grade non-Hodgkin's lymphoma, which was confirmed by histopathologic analysis. The appearance of focal intense hypermetabolic activity of the lesions was different from that of the normal activity in the bowel wall. Rodriguez *et al.*^[4,18] evaluated CT, MRI, and PET in eight patients with primary gastric lymphoma and showed that ^{18}F -FDG PET can demonstrate both the presence and the extent of gastric NHL and is more accurate than endoscopy and CT for evaluating the extent of NHL in the gastric wall. In our study, ^{18}F -FDG PET clearly demonstrated the involvement of specific sites, particularly in the stomach (e.g. the fundus, body, antrum, lesser and greater curvature of the stomach), corresponding to endoscopic biopsy sites. However, extension outside the bowel wall was better appreciated when interpreted with the guidance of a CT scan. It should be noted that an evaluation of the extent of NHL in the gastric wall and careful assessment of multiplicities affect the selection of therapy, since radical surgery with lymph node dissection seems to be needed for most patients with high-grade and even low-grade NHL^[34].

Ullerich *et al.*^[20] and Sam *et al.*^[21] showed that ^{18}F -FDG

PET could find lesions in patients with small bowel lymphoma. Najjar *et al.*^[32] also reported that ^{18}F -FDG PET could identify lymphoma of the colon. These results are consistent with the results in our study, in which pre-treatment ^{18}F -FDG PET showed pertinently high metabolic activity in the small bowel and colon in patients with high-grade NHL (Figure 4). It is difficult to differentiate high-grade non-Hodgkin's lymphoma from other neoplastic or inflammatory diseases. However, interpreting the results in the light of a careful clinical history, the extent of the disease and corresponding CT images, can contribute to the correct diagnosis. Moog *et al.*^[33] showed that ^{18}F -FDG PET imaging could achieve 100% correct diagnoses of malignant lymphoma. In almost all cases of high-grade NHL in our study, CT scans also demonstrated the abnormal wall thickening or mass lesions. However, the results did not reveal the sensitivity of ^{18}F -FDG PET *vs* that of CT, because both modalities were interpreted together as complementary studies.

^{18}F -FDG PET can demonstrate diffuse hypermetabolic activity in patients with low-grade follicular NHL in the colon and stomach. The ability of gallium-67 scintigraphy and ^{18}F -FDG PET to detect MALT-type lymphoma of the gastrointestinal tract is controversial. Hsu *et al.*^[35] showed that gallium-67 scintigraphy could not show abnormal uptake of radioactivity in patients with low-grade gastric MALT-type lymphoma. Hoffmann *et al.*^[19] studied ^{18}F -FDG PET imaging in patients with MALT-type lymphoma and found that PET scans do not show focal tracer uptake in either gastric or extragastric lesions. Our study showed similar results of low activity in the stomach and duodenum. Even though it has been reported that CT scans may demonstrate gastric MALT-type lymphoma either as an infiltrative form or as a polypoid pattern^[36,37], CT is limited in its ability to monitor the treatment response. Taken together, ^{18}F -FDG PET imaging in low-grade NHL seems to be able to monitor the treatment response and determine high-grade transformation.

Generally, in patients with NHL, histologic grade and disease stage are clearly identified as the two major prognostic factors and therapeutic determinants. Endoscopy with biopsy and histopathologic examination

are the gold standard for grading malignancies^[38]. However, there have been attempts to differentiate high-grade from low-grade NHL by different methods (e.g. double-contrast radiography, CT scan and gallium-67 scintigraphy)^[35,39]. ¹⁸F-FDG PET imaging in our study showed that high-grade NHL of the gastrointestinal tract had high FDG activity in the lesions as was confirmed by high SUV measurements. Though the normal background FDG activity in the gastrointestinal tract was known, abnormalities were identified in this study. In low-grade NHL of the gastrointestinal tract, it was difficult to document existing disease, especially in patients with MALT-type lymphoma. However, there was a significant difference in the SUV_{max} measurement between high-grade and low-grade NHL.

This study also demonstrated the clinical utility of ¹⁸F-FDG PET imaging for monitoring patients with NHL of the gastrointestinal tract especially those with high-grade lymphoma. The SUV_{max} measurements were significantly decreased after therapy and post-treatment biopsies were negative for lymphoma. In addition, benign inflammatory conditions such as gastritis showed significantly lower FDG activity. In patients whose post-treatment biopsies were positive for lymphoma, the SUV_{max} measurements were persistently high and even higher in patients with disease progression. Many studies have demonstrated that persistent FDG uptake after therapy may help to predict treatment failure or a high risk of recurrence^[16,17].

In conclusion, ¹⁸F-FDG PET contributes to the diagnosis of high-grade lymphoma involving the gastrointestinal tract, even when there is the normal background FDG activity. Furthermore, the SUV plays a role in evaluating treatment response especially when the PET images are interpreted with CT scans. Low-grade lymphoma demonstrates FDG uptake, but the intensity of the uptake is lower than that in high-grade lymphoma.

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• CLINICAL RESEARCH •

Have patients with esophagitis got an increased risk of adenocarcinoma? Results from a population-based study

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1.25-5.19) and 2.93 (95%CI 0.61-8.59), respectively. In a sensitivity analysis in which all unspecified esophageal cancers were treated as adenocarcinomas, the SIR for adenocarcinoma was 2.64 (0.97-5.75).

CONCLUSION: The risk of adenocarcinoma is not elevated in patients with histological evidence of esophagitis without Barrett's esophagus; however, these patients may have a moderately increased risk of SCC. Further studies are required to confirm these findings, which suggest that Barrett's esophagus, not esophagitis, is the key precursor lesion in the development of adenocarcinoma.

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Key words: Barrett's esophagus; Esophageal adenocarcinoma; Esophageal squamous cell carcinoma; Esophagitis; Population-based study

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Abstract

AIM: To examine an increased risk of esophageal adenocarcinoma is restricted to patients who develop Barrett's esophagus or whether esophagitis *per se* is a risk factor for adenocarcinoma.

METHODS: A population-based cohort of patients with histological evidence of esophagitis without Barrett's esophagus was constructed using electronic pathology reports relating to all esophageal biopsies in Northern Ireland between 1993 and 1996. Person-years of follow-up and incident cases of esophageal cancer were calculated by linking the cohort to death files and the Northern Ireland Cancer Registry records. Standardized incidence ratios (SIR) were calculated for esophageal cancers (adenocarcinoma, squamous cell carcinoma (SCC), and histologically unspecified cancers).

RESULTS: A total of 2 013 patients in the cohort provided 13 559 patient-years of follow-up (mean follow-up 6.7 years). None of the patients developed adenocarcinoma. Three patients developed SCC, and six developed histologically unspecified cancers. The SIR for all esophageal cancers and for SCC were 2.73 (95%CI

INTRODUCTION

The incidence of esophageal adenocarcinoma (OAC) is increasing at a rate faster than any other cancer in the Western world^[1]. The most important risk factor for its development is Barrett's esophagus (BO), a metaplastic condition in which the native squamous epithelium of the esophagus is replaced by columnar epithelium, in response to chronic gastro-esophageal reflux^[2]. OAC appears to result from a sequence of changes from esophagitis to non-dysplastic BO, to dysplastic BO, and finally to adenocarcinoma^[3,4]. It is generally accepted that most, if not all patients who develop OAC, pass through this sequence. It is on this basis that endoscopic surveillance of BO is recommended, in an attempt to reduce mortality from OAC^[5]. However, some aspects of the relationship between BO and OAC remain unclear. While BO is the only known precursor to this tumor, there is a huge variation in the proportion of cases of OAC in which it is detectable, ranging from 23% to 100% of cases in different studies^[6-12]. The difficulty in

identifying BO in patients with OAC is thought to result from tumor overgrowth of the Barrett's segment. However, in a large prospective case-control study in Sweden, Lagergren *et al.*^[13] found that in patients with OAC, only 62% had evidence of BO identified by biopsy, despite extensive sampling by a rigorous protocol. They also found that symptoms of gastro-esophageal reflux *per se* were strongly associated with adenocarcinoma, and that the presence or absence of BO in cancer cases had no effect on the strength of this association. These findings have led to the speculation that gastro-esophageal reflux, rather than BO, is the crucial factor in the development of OAC. If that is the case, then esophagitis may be an important risk factor for its development. To our knowledge, there are no studies assessing the risk of OAC in patients with biopsy-proven esophagitis. We undertook a study to examine the incidence of OAC in a population-based cohort of patients who had histological evidence of esophagitis without BO.

MATERIALS AND METHODS

Patients and biopsies

This study is a follow-up study of a population-based cohort of patients with esophagitis. The cohort comprised every adult within Northern Ireland (NI), population 1.7 million, with histological evidence of inflammation of the esophagus that was not due to infection or radiation. The cohort was constructed by examining pathological reports relating to all esophageal biopsies undertaken within all the hospitals in NI between January 1993 and December 1996. Data were available for biopsies taken between 1993 and 1999, but only the first 4 years were used in order to maximize the period of follow-up. These reports were made available to the NI Cancer Registry (NICR) in an electronic format during the construction of the NI Barrett's Register^[14]. The reports contained information on the nature and site of the submitted biopsy specimen, the clinical summary recorded by the endoscopist on the request form, the full text of the pathologist's report on the specimen, and the pathologist's diagnosis. The clinical summaries contained information relating to the site at which the biopsies were taken. All pathological reports in which the summary stated that the biopsies were taken from the esophagus, including the esophago-gastric junction (OGJ), were examined. A list of SNOMED (Systematized nomenclature of medicine)^[15] codes indicating inflammation in the esophagus was compiled and biopsies were included in the study, if any of these codes appeared on the biopsy report (Table 1). The report which included more than one diagnostic code was considered if at least one of the codes indicated inflammation.

Exclusion criteria for biopsy reports

Biopsy reports were excluded, if patients were less than 16 years of age, or if the diagnostic codes included malignancy, fungal or viral infection, or radiation injury. The bodies of the reports were then examined and any

Table 1 SNOMED diagnostic codes used in study

Code	Diagnostic term	Number of patients
M40000	Inflammation	777
MZ0005	Non-specific inflammation	9
M40005	Active inflammation	88
M41000	Acute inflammation	31
M42100	Active chronic inflammation	62
M43000	Chronic inflammation	279
M76820	Inflammatory polyp	2
M36500	Edema	2
M36100	Congestion	52
M45020	Granulation tissue	11
M14110	Erosion	6
M38000	Ulcer/ulceration	570
MY0102	Reflux esophagitis	125

reports in which the pathologist recorded the presence of columnar epithelium were excluded.

Identification and classification of individual patients

A substantial number of patients in the cohort had esophageal biopsies taken on more than one occasion. Individual patients were identified within the dataset by matching on surname, forename(s), and date of birth (and hospital numbers when they were available). The date of the earliest biopsy showing the inflammation was taken as the date of entry into the cohort.

Further exclusion criteria for patients with columnar epithelium

Some patients had an esophageal biopsy that contained columnar epithelium prior to their first biopsy showing inflammation (NB data only available from January 1993). These patients were excluded from the cohort. Other patients had an esophageal biopsy subsequent to their first biopsy showing inflammation that showed columnar epithelium. These patients were not excluded, unless the biopsy showing columnar epithelium occurred within 3 mo of the initial biopsy. These steps were taken to exclude prevalent cases of BO but to include incident cases in the cohort, the rationale being that if esophagitis leads to malignancy through the development of BO, excluding incident BO would potentially miss these patients.

Follow-up of patients

Members of the cohort were followed up for deaths and incident esophageal cancer OAC, squamous cell carcinoma (SCC), and histologically unspecified cancers and lung cancer till the end of December 2002. Lung cancer incidence within the cohort was determined as a proxy for smoking exposure. Deaths among the cohort were identified by matching with the death files from the Registrar General's Office (NI) using the patient's surname, forename(s), and date of birth. These files contained information on all the deaths that occurred within NI.

Incident cases of esophageal cancer with a date of diagnosis at least 6 mo after the date of entry into the cohort were identified by matching the cohort with the NICR database (using the patient's surname, forename(s), date of birth, and hospital numbers where available). This

population-based cancer registry had collected data on all cancers occurring in NI residents, since the beginning of 1993. When assessing lung cancer incidence, a threshold of 3 mo after the date of entry into the cohort was used.

Statistical methods

Person-years of follow-up were calculated for each member of the cohort with censoring either on the date of diagnosis of the esophageal or lung cancer, on the date of death, or on 31st December 2002. Incidences of OAC, SCC, histologically unspecified esophageal cancers, and lung cancer were calculated as the number of events divided by the person-years of follow-up. These incidences were expressed as events per hundred person-years of follow-up, which is equivalent to percentage incidence per year. The standardized incidence ratio (SIR) for these cancers was calculated by comparing the observed number of cancers in the cohort with the expected number, and by applying the relevant age-specific cancer incidence rates in the NI population to the cohort. Exact confidence intervals (CI) of rates and ratios were estimated using the Poisson distribution. Sensitivity analyses were performed in which histologically unspecified esophageal cancers were reclassified as OACs and then as SCCs and the corresponding SIRs were recalculated. This was done to illustrate the most extreme case scenarios, by assuming that all unspecified cancers were one or the other histological type of cancer. These analyses are secondary analyses of data collected during the construction of the NI Barrett's Register. Establishment of this Register received ethical approval from the Research Ethics Committee of the Queen's University, Belfast.

RESULTS

Figure 1 shows how the cohort was constructed and the effect of exclusion criteria. There were 2 013 patients in the cohort, comprising 996 (49.5%) men and 1 017 (50.5%) women. The mean age was 59.8 years, range 16.2-95.6 years. Men were substantially younger than women (56.6 ± 17.0 vs 63.0 ± 16.2 years). The age and sex distributions of patients are shown in Figure 2. In 90 (4.5%) patients, the biopsies had been taken at the OGJ.

Members of the cohort were followed up for a mean of 6.7 years for a total of 13 559 person-years of follow-up. Nine patients developed esophageal cancer (4 men and 5 women), a mean of 3.1 years (range, 1.0-6.8 years) after the initial biopsy showing esophagitis. Mean age at diagnosis was 75.1 years (range, 60.8-86.3 years). None of the incident esophageal cancers were adenocarcinomas; three were SCCs and six were histologically unspecified. Two of the three patients who developed SCC were women. Histological confirmation of cancer was not possible in five of the six unspecified tumors, as biopsies were not taken. These diagnoses were based on the clinical opinion and in one case the registered causes of death did not include esophageal cancer. The pathological diagnosis in the single unspecified cancer case in whom biopsies had

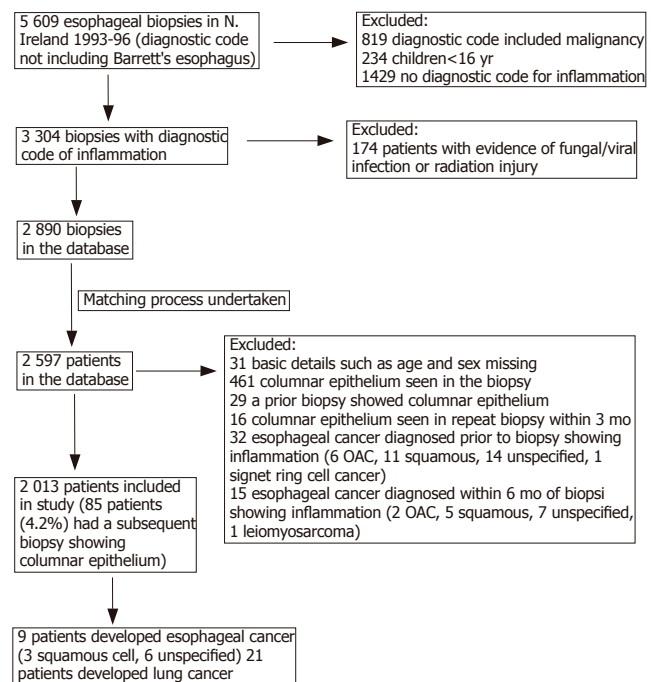


Figure 1 Flow diagram showing how cohort of patients with esophagitis was constructed.

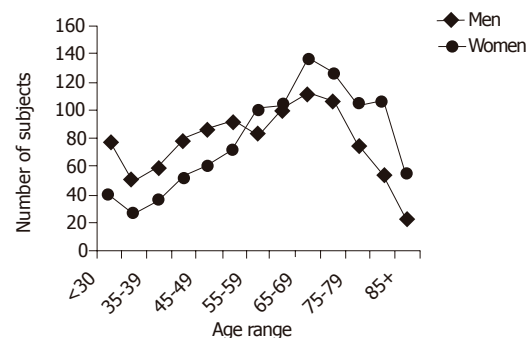


Figure 2 Age and sex distribution of patients with esophagitis.

been taken was recorded as anaplastic carcinoma but in the body of the report the pathologist stated that it was 'probably squamous in type'.

The incidences and SIRs of esophageal cancers are shown in Table 2. The SIR (95%CI) for all esophageal cancers in the cohort was 2.73 (1.25-5.19), with a higher risk in women than men. The SIR (95%CI) for SCC was also raised at 2.93 (0.61-8.59) but this did not reach conventional statistical significance. The SIR for adenocarcinoma was 0 with an upper 97.5%CI of 2.73. None of the esophageal cancers occurred in patients whose biopsies were taken at the OGJ and exclusion of these patients had little effect on observed SIRs (data not shown). In the sensitivity analyses in which histologically unspecified cancers were reclassified as OACs and then as SCCs, the SIRs (95%CI) for OAC and SCC were 2.64 (0.97-5.75) and 4.64 (2.12-8.81), respectively. Dysplasia was noted in 20 (1%) patients; none of these patients

Table 2 Risk of esophageal and lung cancer in patients with histologically confirmed esophagitis and without Barrett's esophagus

		Number of cases	Incidence per 100 person-years of follow-up (95%CI)	Expected number of cases	Standardized incidence ratio (95%CI)
OAC	All patients	0	-	1.35	0 (-, 2.73) ¹
All esophageal cancers	All patients	9	0.07 (0.04, 0.14)	3.29	2.73 (1.25-5.19)
	Men	4	0.06 (0.02, 0.16)	1.8	2.22 (0.61-5.69)
	Women	5	0.07 (0.02, 0.17)	1.43	3.49 (1.14-8.16)
Esophageal SCC	All patients	3	0.02 (0.01, 0.07)	1.02	2.93 (0.61-8.59)
	Men	1	0.02 (0, 0.10)	0.44	2.28 (0.06-12.6)
	Women	2	0.03 (0, 0.12)	0.59	3.40 (0.41-12.2)
Lung cancer	All patients	21	0.16 (0.10, 0.24)	19.71	1.07 (0.66-1.63)
	Men	16	0.25 (0.14, 0.40)	11.36	1.41 (0.81-2.29)
	Women	5	0.07 (0.02, 0.17)	7.63	0.66 (0.21-1.53)
Sensitivity analyses					
Reclassified OAC ²	All patients	6	0.04 (0.01, 0.13)	2.27	2.64 (0.97-5.75)
	Men	3	0.05 (0.02, 0.11)	1.36	2.20 (0.45-6.45)
	Women	3	0.04 (0.01, 0.13)	0.85	3.54 (0.73-10.31)
Reclassified esophageal SCC ³	All patients	9	0.07 (0.02, 0.17)	1.94	4.64 (2.12-8.81)
	Men	4	0.06 (0.02, 0.16)	0.91	4.39 (1.20-11.25)
	Women	5	0.07 (0.02, 0.17)	1.02	4.92 (1.59-11.44)

¹One-sided 97.5%CI; ²OAC+histologically unspecified cancers; ³SCC+histologically unspecified cancers.

developed esophageal cancer.

Eighty-five (4.2%) patients in the cohort had a subsequent esophageal biopsy (after a minimum of 3 mo and before December 1999) showing columnar epithelium. None of the patients developed esophageal cancers in this group.

Twenty-one patients developed lung cancer, including 16 men and 5 women. Mean age at diagnosis of lung cancer was 71.3 years (range, 32.7-80.6 years). Overall, the SIR for lung cancer was not raised although a modest increase in risk was seen in men, which was not statistically significant (SIR 1.41, 95%CI 0.81-2.29).

DISCUSSION

This is the first population-based study to assess the risk of esophageal cancer in patients with histologically proven esophagitis that is not complicated by BO. We found that none of the 2 013 patients with esophagitis developed OAC after an average follow-up of 6.7 years. However, some of the six histologically unspecified tumors that occurred within the cohort might be adenocarcinomas.

This study has got many advantages. All patients had histologically confirmed esophagitis, and every patient in whom this diagnosis was made in NI between 1993 and 1996 was included in the study. The population-based nature of this study avoids selection biases that may operate when only patients attending specific centers are investigated. Also, patients who showed evidence of BO were excluded from the cohort, which allowed us to examine the risk of esophageal cancer in esophagitis uncomplicated by BO. Finally, follow-up of patients is likely to be near-completion for two reasons: firstly, population-based cancer and death registers were used to determine deaths and cancer incidence among the cohort; and secondly, emigration from NI is uncommon, averaging 1.1% of the population per year in the period 1991-2003^[16].

This study also has limitations. A diagnosis of 'reflux esophagitis' was made in only a small proportion of cases because scant clinical details were provided on the pathology request forms. However, reflux of gastric contents was the most likely cause of esophagitis that was not due to infection or radiation. Also, patients with esophagitis were not routinely biopsied, so patients included in this cohort may represent a subset of patients with esophagitis who had suspicious features at endoscopy. Risk of esophageal cancer may, therefore, be exaggerated in this cohort but exclusion of prevalent cancers and cancers occurring within the first 6 mo reduces the effect of this problem. Accurate histological classification of the incident esophageal tumors occurring in the cohort was not available for 60% of tumors. Some of the unclassified tumors may be adenocarcinomas rendering the absolute incidence of OAC, an underestimate. However, classification of esophageal cancer incidence in the general population is subject to the same limitations, meaning that the SIRs presented for all cancers and for cancer subgroups should be valid comparisons between the cohort and the general population. Furthermore, we performed sensitivity analyses to estimate the maximum incidence of OAC and SCC, by assuming that all the histologically unspecified cancers were actually OACs or SCCs, respectively. Lastly, the modest size of the cohort and the period of follow-up have resulted in small numbers of incident cancers, which may render some of the estimates of cancer risk unstable. Another effect of the relatively short period of follow-up is that a late increase in the risk of OAC associated with esophagitis cannot be discounted.

When all histologically unspecified cancers were treated as OACs, giving a maximal possible risk of OAC, the risk in the cohort was approximately 2.5 times than that of the general population. This is undoubtedly an overestimate but the extent cannot be ascertained. We have previously demonstrated a 17-fold increase in risk of OAC in a cohort

of patients with BO (histologically confirmed specialized intestinal metaplasia) drawn from the same population and followed up in the same manner as in this study^[14]. These data suggest that, unlike BO, esophagitis without BO is not an important risk factor for OAC.

The literatures regarding the risk of adenocarcinoma in esophagitis are conflicting. Previous case-control studies have shown that esophagitis or esophageal ulcer is associated with a two- to fivefold increased risk of OAC^[17-19]. However, the diagnosis of esophagitis was obtained from case-note review or questionnaire and was not histologically confirmed. Also, it was not possible to identify and exclude patients with BO in these studies. Ye *et al.*^[20] reported a sixfold increase in the incidence of OAC in a large population-based cohort study of patients with a hospital diagnosis of gastro-esophageal reflux disease (GORD), but patients with BO were not excluded. A recent study utilizing data from the United Kingdom General Practice Research Database estimated that patients with esophagitis had a 4.5-fold increased risk of OAC compared to the normal population, whereas the risk in BO patients was increased 30-fold^[21]. Moreover, following long-term follow-up of patients with GORD, Spechler *et al.*^[22] found that patients with BO at baseline developed OAC at an annual rate of 0.4%, whereas the rate in patients without BO was only 0.07%. These data from patients with clinical diagnoses of esophagitis and BO appear to be in agreement with our findings from patients with histological confirmation of their diagnoses.

Only 4.5% of patients in our cohort had a subsequent esophageal biopsy showing columnar epithelium, suggesting that progression from esophagitis to BO may be uncommon. However, it must be borne in mind that there was no systematic recall of patients for endoscopy, so the incidence of BO in the cohort may be underestimated. Also, a systematic biopsy protocol was not employed, so columnar epithelium may have been missed in some patients, either in their initial biopsy or subsequent biopsies.

The low incidence of OAC in our cohort suggests that progression along a path from esophagitis to BO and to OAC may not be the normal sequence of events. Rather, an individual's response to reflux of gastric contents may result in either esophagitis or BO, with a future risk of OAC being confined to those who develop BO. Cameron and Arora^[23] carried out a detailed study in which they mapped areas of damage seen at endoscopy in patients with esophagitis and in patients with BO. Their findings suggest that BO is unlikely to develop directly from areas of esophagitis, adding plausibility to the proposed pathway in Figure 3.

The incidence of SCC within the cohort was almost three times than that of the general population. There is limited evidence in the literature supporting a link between esophagitis and SCC. Munoz *et al.*^[24] showed a very high prevalence of esophagitis (84%) in people living in the Linxian region of China, where there is a very high incidence of esophageal SCC. However, the authors stated that this esophagitis affected the middle and lower thirds

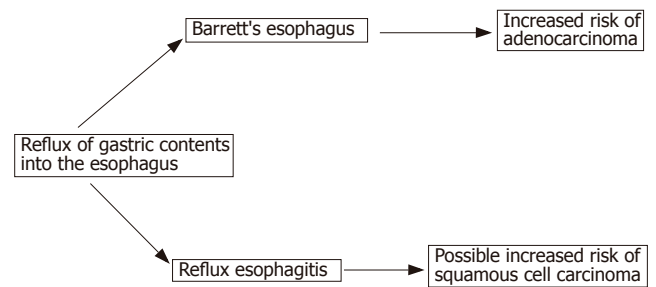


Figure 3 Potential pathways of cancer causation in the esophagus.

of the esophagus as opposed to the distal esophagus; also, it was not accompanied by erosions or ulcers, features characteristic of esophagitis secondary to gastro-esophageal reflux. These patients are very different from the cohort that we had examined, particularly in terms of lifestyle and nutritional status and gastro-esophageal reflux may not be important in the etiology of esophagitis in China. Ribet *et al.*^[25] found evidence of an association between reflux symptoms and SCC in the patients who underwent resection of esophageal cancers, but this was a small study involving a highly selected group. Ye *et al.*^[20] found a moderately raised risk of SCC in a Swedish cohort of hospital patients with GERD. On the other hand, in a large Swedish case-control study, Lagergren *et al.*^[13] found no association between symptoms of reflux and risk of SCC of the esophagus.

Our finding of increased SCC risk in patients with esophagitis could result from over-representation of smokers within the cohort, since smoking is associated with reflux symptoms^[26,27]. However, this was unlikely because the risk of lung cancer was not raised in the cohort. It may also result from inclusion of patients with suspicious lesions seen at endoscopy, which might include dysplastic squamous epithelium; however, less than 1% of patients had evidence of dysplasia, and none of these developed SCC. Further research is required to examine the association we have seen between esophagitis and SCC.

In summary, our study shows that esophagitis that is not complicated by BO is not associated with an increased risk of OAC. This finding supports the view that BO, not esophagitis, is the key precursor of OAC. Further studies are required to see if these findings can be replicated. Determination of the relative importance of esophagitis in esophageal cancer causation is important because this has implications for the management of patients with gastro-esophageal reflux and for the prevention of OAC.

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• CLINICAL RESEARCH •

Ultrasonographic study of postcibal gastro-esophageal reflux and gastric emptying in infants with recurrent respiratory disease

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Abstract

AIM: To check the utility of postcibal ultrasonography for the evaluation of reflux in relation to gastric emptying in infants with recurrent respiratory symptoms and to link imaging with clinical data.

METHODS: Esophageal reflux (hyperechoic retrograde filling) and gastric emptying (antral areas) were quantified before and after ingestion of a standard formula in 35 untreated infants (13 with chronic cough, 22 with recurrent bronchitis) and in 31 controls.

RESULTS: The prevalence of abnormal (≥ 8 episodes) postcibal refluxes was 74% in patients and 3% in controls. Number, duration of the longest episode and extent of refluxes were significantly higher in patients compared to controls. Number of refluxes was higher in patients with symptomatic refluxes than in those without. Infants with recurrent bronchitis had more refluxes than those with chronic cough and controls. Extent and timing of gastric emptying were similar in patients and controls.

CONCLUSION: Esophageal ultrasonography is a useful and physiological test in infants with recurrent respiratory diseases, which have a high prevalence of abnormal postcibal esophageal reflux and a gastric emptying similar to that of normal controls. Esophageal reflux is more severe in subjects with recurrent bronchitis than in those with chronic cough.

INTRODUCTION

Gastro-esophageal reflux has been frequently linked to several acute and chronic respiratory diseases in infants^[1-4]. As ultrasonography may allow direct visualization of gastro-esophageal junction and of retrograde reflux episodes^[5,6], it has been used as an alternative to invasive techniques in pediatric age^[5-9]. Validation studies reported high sensitivity and specificity for ultrasonography in diagnosing gastro-esophageal reflux, as compared to standard barium swallow examination^[6], esophageal pH monitoring^[8,10], and esophagomanometry^[10]. The application of ultrasonography in pediatric age has major advantages due to its non-invasiveness, wide availability, and optimal compliance. Thus, in the present study, we aimed to: (1) employ ultrasound for the evaluation of gastro-esophageal reflux in relation to postprandial gastric emptying in infants with or without recurrent respiratory diseases; and (2) relate the gastro-esophageal imaging with clinical data in the postcibal period.

MATERIALS AND METHODS

Subjects

The ultrasonographic test was part of a routine work-up abdominal assessment. Informed consent was obtained from parents for each infant participating in the study and the study protocol was approved by an institutional review board. The study comprised 66 non-preterm infants (age range, 1-12 mo). Characteristics of the subjects are summarized in Table 1. Thirty-five inpatient or outpatient infants with a recent history of recurrent respiratory diseases were recruited. Patients had at least two episodes of asthmatic bronchitis requiring hospital admission (group A, $n = 22$) or unexplained chronic cough (group B, $n = 13$). Patients, who were referred only for respiratory problems, underwent *de novo* evaluation without prior investigations or therapies. Parents denied, in all cases, the consent to

invasive techniques. In all patients, immune deficiencies, cystic fibrosis, and allergopathies were excluded by clinical and biohumoral assessment. Parents were asked for the presence of daily regurgitation in all the patients.

Additionally, 31 inpatient or outpatient infants, who never experienced respiratory or gastrointestinal diseases, were considered as controls. Patients were not examined during acute respiratory disease. Neither patients nor controls were under medications able to influence gastrointestinal motility or secretion and were not affected by conditions known to alter gastrointestinal tract functions.

Study design

Each subject underwent a simultaneous ultrasonographic study of the esophagus and of the antrum with a Toshiba Capasee scanner and a 3.5-MHz convex probe. Ultrasonography was performed in a sitting position before and after the ingestion of a low volume liquid standard meal (10 mL/kg standard infant formula). Subjects were fed in the upright position. The observation was carried out continuously for at least 20 min and in all cases until complete antral evacuation was recorded. All tests started in the morning, in the fasting state (at least 4 h) and were performed by a single examiner who was unaware of patient's history and clinical examination. Ultrasonographic findings were analyzed while being done and events were recorded on a specific form. The infants were not burped after the feeding and pacifiers were not employed. All infants were awake during the examination. Subjects with excessive gas in the stomach or excessive crying time (able to interrupt the continuity of observation) were excluded by analysis due to technical limitations.

Gastro-esophageal reflux study

The employment of ultrasound in the diagnosis of gastro-esophageal reflux disease has previously been validated against other techniques^[6,8,10]. The esophagus and the gastro-esophageal junction were studied by transverse and longitudinal scans at the epigastrium^[5,6,11] at the level of the left lobe of the liver, slightly rotating up the probe. In all the subjects, this allowed the visualization of the esophagus like a three-layer canalicular structure posterior to the left lobe of the liver and anterior to the aorta, which were used as landmark points^[11]. Immediately after the ingestion of the standard liquid meal, the esophageal lumen appeared dilated by hyperechoic content flowing towards the gastric lumen. First, a longitudinal scan was performed to show the emptying of the distal esophagus. Subsequently, a "real time" observation of the esophagus and the gastro-esophageal junction was performed^[11] by splitting the image on the ultrasound monitor. In particular, the esophagus and the gastro-esophageal junction were imaged on one half of the monitor continuously during 20 min. At regular intervals (mentioned below), the observation was stopped shortly for 2-3 s to catch antral sections on the second half of the screen for subsequent antral area measurement.

Gastro-esophageal reflux, when present, was visible like

a flow of hyperechoic material directed from gastric lumen to distal esophagus through gastro-esophageal junction.

In the present series, gastro-esophageal reflux was defined as abnormal when the total number of episodes lasting for more than 2 s was ≥ 8 (upper limit derived from mean+2SD in normal control group = 7.5). The presence/absence and the number of gastro-esophageal reflux episodes during 20 min were recorded and the frequency of gastro-esophageal reflux episodes (i.e., no. of episodes/time unit) was therefore calculated.

Immediately after the retrograde flow of hyperechoic material from gastric lumen to distal esophagus, the ultrasonographic image was frozen on one half of the monitor and the amount of gastro-esophageal reflux was subsequently quantified by measuring maximal esophageal lumen enlargement (distance between external esophageal layers). On the second half of the monitor (set as "real time" imaging), the duration of the reflux episode was counted by a timer. Maximal esophageal lumen enlargement and duration of the longest reflux episode for each examination were noted.

During the real-time continuous monitoring, all cough episodes were counted in both patients and controls. Cough episodes starting within 2 s from hyperechoic retrograde filling of esophageal lumen were therefore marked on a specific form and were considered related to reflux.

Gastric emptying study

Postprandial evacuation of the stomach was assessed by functional ultrasonography by monitoring antral areas, a well standardized technique in adults^[11-15] and in infants^[16-20]. Antral area was quantified in fasting infants and subsequently monitored at regular time intervals postprandially by longitudinal scans at the epigastrium at the level of antral-body connection in a single section. The superior mesenteric vein and the aorta were used as landmark points. Antral emptying was described by the following indices: (1) antral (basal) area (mean of two measurements between -5 and 0 min before meal, in cm²); (2) maximal postprandial antral area, measured 2 min after meal ingestion; (3) postprandial antral areas each taken at 5-min intervals from meal ingestion; (4) minimal postprandial antral area observed throughout the emptying curve; and (5) half-emptying time.

Antral emptying curves were obtained by plotting antral areas *vs* time. In order to obviate interindividual variability of antrum size^[13], postprandial areas were normalized to maximal areas after subtracting basal areas: $100 \times (A_t - a) / (A_2 - a)$, where A_t = postprandial area at any given time; a = basal area; A_2 = maximal antral area (measured at 2 min postprandially)^[21]. Half-emptying time was the time at which 50% decrease of antral area occurred ($T_{1/2}$, min), and was calculated by linear regression analysis from the linear part of antral emptying curve. This index closely correlates with the scintigraphic half-emptying time^[14].

Statistical analysis

All calculations were performed with the NCSS2001

Table 1 Characteristics of enrolled subjects

	Patients	Controls	P
N	35	31	
Age (mo)	5.6±2.9	4.5±3.6	NS
Weight (kg)	7.4±2.0	6.7±1.7	NS
Height (m)	0.7±0.07	0.6±0.01	NS

Data are expressed as mean±SD. NS: not significantly different.

Table 2 Ultrasonographic characteristics of gastro-esophageal reflux in infants with recurrent respiratory diseases and in healthy controls

	Patients	Controls	P
Number of reflux episodes	10 (2-21)	3 (1-9)	<0.01
Reflux frequency (reflux episodes/min)	0.5 (0.1-1.05)	0.15 (0.05-0.45)	<0.01
Maximal esophageal lumen enlargement (mm)	5 (1-17)	1.5 (1-9)	<0.01
Duration of the longest reflux episode (s)	11 (2-18)	4 (2-12)	<0.01

Results are expressed as median (range). Differences between groups were tested using Mann-Whitney *U* test.

software (Kaysville, UT, USA). Results were expressed as mean±SE or mean±SD or median and range, where appropriate. Data were statistically compared using two-tailed Student's *t*-test for unpaired data or by non-parametric Mann-Whitney *U* test, where appropriate. Analysis of variance followed by Fisher's LSD multiple comparison test was utilized to compare the differences among the groups. The χ^2 test was used to compare proportions of categorical data. Two-tailed *P* values less than 0.05 were considered statistically significant^[22].

RESULTS

Patients and controls were similar in age-weight and height distribution (Table 1) and all subjects were below the 95th percentile for body weight. Overall, patients had significantly greater prevalence of abnormal postcibal reflux (74% in patients *vs* 3% in controls, *P*<0.05), number and frequency of reflux episodes, extent of refluxes and duration of the longest reflux episode as compared to controls (Table 2). Stratification of patients by age (0-6 mo, *n* = 22 and 7-12 mo, *n* = 13) showed similar results in the two age groups that were examined (Table 3).

The total number of cough episodes was significantly greater in patients than in controls (2.7±0.5 *vs* 0.03±0.03; *P*<0.01). Symptomatic reflux episodes (i.e., cough observed within 2 s from hyperechoic retrograde filling of esophageal lumen) were documented in 57% of patients (2.0±0.4 symptomatic refluxes in the observation period) and in none of the controls. Cough episodes not preceded by reflux were 0.5±0.3 in patients without symptomatic reflux episodes and 0.8±0.2 in patients with symptomatic refluxes.

The median number and frequency of gastro-esophageal reflux episodes were obviously higher in patients with symptomatic reflux episodes than in those without (Table 4).

Table 3 Ultrasonographic characteristics of gastro-esophageal reflux in patients with recurrent respiratory diseases in two age groups

	0-6 mo	7-12 mo	P
Number of reflux episodes	9.5 (3-21)	11 (2-18)	NS
Reflux frequency (reflux episodes/min)	0.5 (0.15-1.05)	0.6 (0.1-0.9)	NS
Maximal esophageal lumen enlargement (mm)	5.5 (1-17)	4 (1.6-11.5)	NS
Duration of the longest reflux episode (s)	11 (2-17)	7 (4-18)	NS

Results are expressed as median (range). Differences between groups were tested by Mann-Whitney *U* test; NS: not significantly different.

Table 4 Ultrasonographic characteristics of gastro-esophageal reflux in patients with or without symptomatic reflux episodes

	With symptomatic reflux	Without symptomatic reflux	P
Subjects	20	15	
Number of reflux episodes	11.5 (4-21)	7 (2-18)	<0.02
Reflux frequency (reflux episodes/min)	0.58 (0.2-1.05)	0.4 (0.1-0.9)	<0.05
Maximal esophageal lumen enlargement (mm)	5.5 (1.7-13.5)	3.0 (1-17)	NS
Duration of the longest reflux episode (s)	11 (6-18)	8 (2-17)	NS

Results are expressed as median (range). Differences between groups were tested using Mann-Whitney *U* test. NS: not significantly different.

The prevalence of abnormal refluxes was higher in patients with recurrent bronchitis (group A) and in patients with chronic cough (group B) as compared to controls (Table 5). Both subgroups of patients had more reflux episodes, higher reflux frequency, higher esophageal lumen enlargement and duration of the longest reflux episode as compared to controls (*P*<0.01, ANOVA, Figure 1 and Table 5). Patients with recurrent bronchitis also showed more reflux episodes, higher reflux frequency and higher duration of the longest reflux episode as compared to infants with chronic cough (Table 5). The number of symptomatic reflux episodes was similar in patients with recurrent bronchitis (2.6±0.6) and in those with chronic cough (1.2±0.4).

Daily regurgitation was present in 57% of patients with recurrent respiratory diseases, who showed a higher number, frequency, and duration of the longest reflux episode compared to patients without this symptom (Table 6). The number of symptomatic refluxes was similar in patients with (2.4±0.6) and without (1.7±0.6) daily regurgitation.

The study of gastric emptying showed a rapid and continuous decrement of the antral areas starting immediately after meal ingestion in both patients and controls (Figure 2). Patients and controls showed comparable fasting (1.3±0.01 and 1.2±0.1 cm², respectively) and maximal postprandial areas (5.7±0.3 *vs* 5.6±0.4 cm²), and similar postprandial emptying indices, in terms of both minimal postprandial areas (1.4±0.1 *vs* 1.3±0.2 cm²; 4.6±1.9% *vs* 3.5±1.4%) and half-emptying time (9±0.6 *vs* 9±0.5 min, respectively). No significant difference was observed in time to maximal emptying between patients

Table 5 Ultrasonographic characteristics of gastro-esophageal reflux in patients with recurrent episodes of bronchitis (group A), chronic cough (group B), and in healthy controls

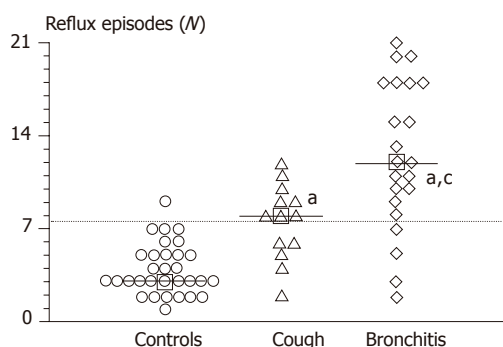
	Group A	Group B	Controls	ANOVA
Reflux prevalence (%)	82 ^a	54 ^a	3	
Number of reflux episodes (N)	12.0 (2-21) ^{a,c}	8.0 (2-12) ^a	3.0 (1-9)	<0.00001
Reflux frequency (N/min)	0.6 (0.1-1.05) ^{a,c}	0.4 (0.1-0.6) ^a	0.15 (0.05-0.45)	<0.00001
Maximal esophageal lumen enlargement (mm)	5 (1-17) ^a	4 (1.5-11.8) ^a	1.5 (1-9)	<0.0001
Duration of the longest reflux episode (s)	11.5 (2-18) ^{a,c}	7 (3-12) ^a	4 (2-12)	<0.00001

Results are expressed as median (range). Differences between percentage reflux prevalence were tested by χ^2 test. Differences between groups were examined by using ANOVA, followed by Fisher's LSD multiple comparison test. ^a $P < 0.05$ vs controls; ^c $P < 0.05$ vs group B.

Table 6 Ultrasonographic characteristics of gastro-esophageal reflux in infants with recurrent respiratory diseases, with or without daily regurgitation

	With daily regurgitation	Without daily regurgitation	P
Subjects	20 (57%)	15 (43%)	NS
Number of reflux episodes (N)	10.5 (5-21)	8 (2-18)	<0.03
Reflux frequency (N/min)	0.5 (0.25-1.05)	0.4 (0.1-0.9)	<0.03
Maximal esophageal lumen enlargement (mm)	5 (1.5-17)	4 (1.0-11.5)	NS
Duration of the longest reflux episode (s)	11.5 (3-17)	7 (2-18)	<0.01

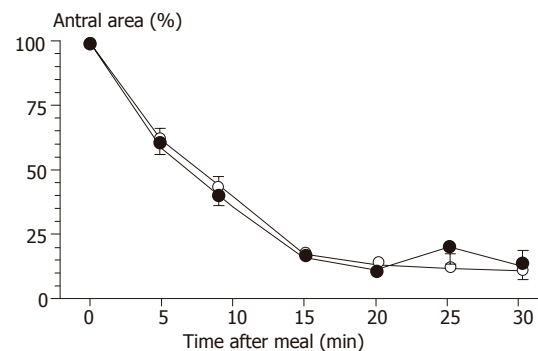
Results are expressed as median (range). Differences between groups were tested by using Mann-Whitney *U* test. NS: not significantly different.

**Figure 1** Number of postcibal gastro-esophageal reflux episodes, as determined by ultrasound, in controls ($n = 31$) and in infants with recurrent respiratory diseases ($n = 35$). Horizontal dotted line indicates the mean+2SD in the normal control group. Squares and continuous horizontal lines indicate medians. Differences between the groups were tested by using ANOVA, followed by Fisher's LSD multiple comparison test. ^a $P < 0.05$ vs controls; ^c $P < 0.05$ vs chronic cough.

(20 ± 1 min) and controls (19 ± 1 min). Invariably, gastric emptying was complete after 30 min in all cases. Gastric emptying speed was also similar in patients with and without symptomatic reflux episodes (half-emptying time 9 ± 1 and 10 ± 1 min, respectively) and in patients with and without daily regurgitation (half-emptying time 9 ± 1 and 9 ± 1 min, respectively).

DISCUSSION

The present study shows that ultrasonography is useful in providing important information about the presence, extent, and duration of postcibal gastro-esophageal reflux

**Figure 2** Postcibal gastric emptying curves, determined by ultrasound, in controls ($n = 31$) and in infants with recurrent respiratory diseases ($n = 35$). Symbols indicate means (\circ : controls; \bullet : patients), and vertical lines indicate SE. Differences between groups were tested by using ANOVA.

in infants with recurrent respiratory diseases.

Gastro-esophageal reflux is the effortless retrograde flow of gastric content in the esophageal lumen and, when abnormal, it is a recognized clinical problem in infancy, where it has been frequently associated with respiratory diseases^[1-4,23].

Respiratory diseases and gastro-esophageal reflux disease have long been linked in childhood by both physiopathological and epidemiological studies, and the complex interplay between these two frequent clinical conditions is still under debate^[24,25].

By prolonged pH-metry, a high prevalence of abnormal esophageal pH was reported in infants with recurrent bronchopulmonary infections, suggesting that this technique may be useful for diagnosing gastro-esophageal reflux and its association with recurrent bronchopulmonary diseases^[26]. pH-metry, however, has several pitfalls, since it does not help with alkaline gastric content as seen in a number of conditions, namely after the ingestion of normal infant formula, achlorhydria or duodenogastric reflux^[27,28], and alkaline reflux episodes^[29,30]. In a recent study, intraluminal impedance has been used in infants with respiratory phenomena and it has been shown that gastro-esophageal refluxes accompanied by respiratory symptoms can be detected only in a minority of cases by pH-metry^[29]. Similar findings were also evident in a large ultrasonographic study in symptomatic infants undergoing pH-metry^[7].

Impedance monitoring is a pH-independent method for detecting bolus movement within the esophagus.

It might provide a valid alternative to esophageal pH-metry because it evaluates reflux occurring both in the interprandial and postprandial period, and it is sensitive to very small volumes of refluxate^[29-33].

On the other hand, functional ultrasonography of the gastro-esophageal junction may allow direct visualization of lower esophageal sphincter relaxation and reflux episodes. The technique offers several advantages in that it has low costs, it is simple, non-invasive and is a more physiological alternative to invasive techniques^[5,6,8-10]. In this respect, our results suggest that ultrasonography might represent a useful and physiological screening test in infants with recurrent respiratory diseases.

The importance of meal volume also needs to be addressed. We used a relatively low-volume liquid standard meal (i.e. of lower volume compared to that currently used during a physiological feeding). Such low volume would not affect the observed results, since in early studies, gastro-esophageal reflux was either unrelated to feed volume^[17] or it was worsened by a high volume meal, through significant changes in lower esophageal sphincter pressure^[20]. Here, we have observed several postcibal refluxes with a low-volume meal, suggesting that meal volume *per se* may not be a determinant in postcibal reflux occurrence.

The short postprandial observation period might be considered as a major pitfall of the ultrasonographic study. However, by using a 20-min postprandial time-window to check for persistence of intragastric content, we found significant differences for extent and timing of gastro-esophageal reflux between patients with recurrent respiratory diseases and controls, and an event/symptom relationship. Indeed, patients with recurrent involvement of lower respiratory tract (i.e. bronchitis) showed a higher number of postcibal reflux episodes compared to both patients with chronic cough and controls.

While interprandial refluxes could not be documented at ultrasonography, we underlined the importance of postcibal reflux in the pathogenesis of recurrent respiratory diseases in infants. Transient lower esophageal sphincter relaxation is the predominant mechanism of gastro-esophageal reflux^[34] and postprandial reflux has been suggested to be a dynamic indicator of sphincter competency^[20,35]; in this respect, postcibal ultrasonography allows direct visualization of both gastro-esophageal reflux and sphincter incompetency.

It has been reported that delayed clearance of refluxed material and clustering of reflux events may precipitate respiratory symptoms in infants^[36,37]. Indeed, we found that amount, extent and number of postcibal gastro-esophageal reflux episodes were higher in subjects with symptomatic refluxes than in those without.

Although ultrasound visualization of reflux is limited to the distal esophagus, our results suggest the possibility that the involvement of the respiratory tract might be partly due to a greater chance for microaspiration of refluxed materials reaching the proximal esophagus. This possibility is more likely in subjects with a great amount and timing of gastro-esophageal reflux episodes. Interestingly, a

recent study found a direct relation between incidence and duration of refluxes in the proximal and in the distal esophagus^[38]. However, other mechanisms responsible for respiratory tract involvement secondary to gastro-esophageal reflux, particularly vagally mediated reflexes, may be taken into account^[39].

The association of gastro-esophageal reflux disease and chronic cough in infants has been well demonstrated^[2,23,40], and our results suggest that postcibal ultrasonography might be employed as a useful diagnostic approach also in patients with chronic cough.

Delayed gastric emptying has been frequently linked to gastro-esophageal reflux both in adults^[41-43] and in pediatric age^[16,44]. In a scintigraphic study in pediatric patients, it was found that delayed gastric emptying increased postprandial reflux by increasing the refluxate volume per episode of reflux through an underlying incompetent lower esophageal sphincter^[44]. By using ultrasonography, we also performed the measurement of gastric emptying by a well standardized and validated procedure^[11,16-20]. Our data demonstrated a similar extent and timing of postprandial gastric emptying in patients and controls. This is in accordance with a previous ultrasonographic study in infants with reflux disease, in which the speed of gastric emptying was unrelated to any interprandial or postprandial indices of gastro-esophageal reflux by a 24-h pH-metry^[17]. Similar conclusions were also depicted by another recent study by (13)C-octanoic acid breath test, showing that gastric emptying was not delayed in infants with gastro-esophageal reflux^[34].

In conclusion, postcibal esophageal ultrasonography is a useful and physiological screening test in patients with recurrent respiratory diseases, who show lower esophageal sphincter incompetence and high postprandial gastro-esophageal reflux prevalence, with a postprandial gastric emptying similar to controls. Subjects with recurrent bronchitis seem to have a greater amount of gastro-esophageal reflux episodes compared to those with chronic cough.

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Human leukocyte antigen class II DQB1*0301, DRB1*1101 alleles and spontaneous clearance of hepatitis C virus infection: A meta-analysis

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infection. Large, multi-ethnic confirmatory and well-designed studies are needed to determine the host genetic determinants of HCV infection.

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Key words: Human leukocyte antigen; Genetic polymorphism; DQB1*0301; DRB1*1101; Hepatitis C virus; Spontaneous clearance; Meta-analysis

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Abstract

AIM: To assess the associations of human leukocyte antigen (HLA) class II DQB1*0301 and/or DRB1*1101 allele with spontaneous hepatitis C virus (HCV) clearance by meta-analysis of individual dataset from all studies published till date.

METHODS: To clarify the impact of HLA class II polymorphisms on viral clearance, we performed a meta-analysis of the published data from 11 studies comparing the frequencies of DQB1*0301 and DRB1*1101 alleles in individuals with spontaneous resolution to those with persistent infection. As we identified the heterogeneity between studies, summary statistical data were calculated based on a random-effect model.

RESULTS: Meta-analyses yielded summary estimates-odds ratio (OR) of 2.36 [95%CI (1.62, 3.43), $P < 0.00001$] and 2.02 [95%CI (1.56, 2.62), $P < 0.00001$] for the effects of DQB1*0301 and DRB1*1101 alleles on spontaneous clearance of HCV, respectively.

CONCLUSION: These results support the hypothesis that specific HLA class II alleles might influence the susceptibility or resistance to persistent HCV infection. Both DQB1*0301 and DRB1*1101 are protective alleles and present HCV epitopes more effectively to CD4⁺T lymphocytes than others, and subjects with these two alleles are at a lower risk of developing chronic HCV

INTRODUCTION

HCV infection presents with diverse clinical manifestations. Approximately 20% of infected patients successfully eliminate the virus, whereas the majority of patients develop chronic infection with a wide spectrum of liver lesions, ranging from minimal inflammation to cirrhosis and hepatocellular carcinoma (HCC)^[1]. The mechanisms underlying the spontaneous viral clearance or development of chronic HCV infection have not yet been identified^[2]. Apart from viral characteristics (viral genotype, quasi-species distribution and viral load), it is generally accepted that cellular immune responses play an important role in viral clearance and disease resolution^[3]. Clearance of the virus during acute infection has been shown to be associated with strong and sustained HCV-specific CD4⁺ and CD8⁺ T-cell responses^[4-8]. Though CD8⁺ T cells are primary effector cells directly eliminating HCV-infected cells, CD4⁺ T cells play a central regulatory role, which modulates the CD8⁺ T-cell responses and is crucial for the production of neutralizing antibodies. A number of recent studies have suggested that early sustaining vigorous and multi-specific CD4⁺ T-cell responses might be a critical determinant for the primary HCV infection^[9,10]. The importance to CD4⁺ T-cell response is the presentation of HCV antigens in the context of HLA class II molecules. Therefore, the differences in HLA class II alleles may strongly influence the clinical course of hepatitis C.

HLA genes located on chromosome 6 encode peptides

Table 1 Characteristics of studies included in the meta-analysis

First author (year) [reference]	Country (ethnicity)	Spontaneous resolution	Persistent infection	Matching	Anti-HCV tests	HLA typing
Alric (1997) [21]	France (European)	25, M/F: 9/16 Age: 40.6±15.7 yr	103, M/F: 58/45 Age: 45.4±12.4 yr	Sex, age, source of HCV infection, HCV-serotype	2G EIA and RIBA	PCR-SSOP
Cramp (1998) [22]	UK (European)	49, M/F: 30/19 Duration: 15.5 (3-42) yr	55, M/F: 31/24 Duration: 14.2 (2-40) yr	Sex, age, source of HCV infection and duration	2G line immunoassay	PCR-SSOP
Minton (1998) [23]	UK (European)	35, M/F: 19/16 Age: 37.9±10.8 yr	138, M/F: 87/51 Age: 37.2±10.1 yr	Sex, age, source of HCV infection	2G ELISA and RIBA	PCR-SSOP
Mangia (1999) [24]	Italy (European)	35	149	Sex, HCV-serotype, not age, not duration	RIBA and 3G EIA	PCR-SSP
Thursz (1999) [25]	European	85, M/F: 37/48 Age: 45±14 yr	170, M/F: 74/96 Age: 50±16 yr	Sex, center, not age	ELISA and RIBA	PCR-SSP
Vejbaesya (2000) [26]	Thailand (Asian)	43 Blood donor M/F: 25/18	57 M/F: 31/18	Sex	2G EIA and RIBA	PCR-SSOP
Alric (2000) [27]	France (European)	63, M/F: 21/42 Age: 42.1±15.4 yr	282, M/F: 150/132 Age: 46±12.3 yr	Age, source of HCV infection and duration, not sex	2G EIA and RIBA	PCR-SSOP
Fanning (2000) [13]	Irish (European)	85 Female	72 Female	From single source	RIBA	Reverse line probe hybridization
Thio (2001) [28]	North America	200, M/F: 166/34 Age: 25.7 yr	374, M/F: 310/64 Age: 27.8 yr	Age, sex, race	2G EIA and RIBA	PCR-SSP PCR-SSCP
Azocar (2003) [29]	Hispanic (European)	40, M/F: 33/7 Age: 37.9 yr	72, M/F: 54/18 Age: 39.2 yr	Age, sex	EIA and RIBA	PCR-SSOP PCR-SSP
Spada (2004) [30]	Italy (European)	10, M/F: 5/5 Age: 40.5 (20-61) yr	24, M/F: 22/2 Age: 29 (20-56) yr	Not sex, age, source of HCV infection, HCV-serotype	3G ELISA and RIBA	PCR-SSP

EIA: enzyme-immunoassay; RIBA: recombinant immunoblot assay; ELISA: enzyme-linked-immunosorbent assay; 2G: second generation; 3G: third generation; SSOP: sequence-specific oligonucleotide probes; SSP: sequence specific primers; SSCP: single stranded conformational polymorphisms.

involved in host immune response. Moreover, the HLA loci display an unprecedented degree of diversity in human genome, presumably an evolutionary adoption to immune pressure from various infectious agents. Polymorphisms in HLA class II molecules can give rise to amino acid substitutions with different peptide-binding characteristics and determine antigenic specificities, the strength of immune response to HCV^[11,12]. Consequently, distinct HLA class II alleles may be expected to exert an impact on the development of host immune responses against HCV infection. However, few studies have demonstrated consistency in different populations. In several Irish studies performed in women exposed to HCV genotype 1b from a single inoculum, DRB1*0101 and DQB1*0501 alleles are found to be associated with viral elimination^[13-17]. One German study suggests that HLA-DR15 (B1*15011) might constitute an important genetic factor for the clearance of HCV^[18]. Thio *et al.*^[19] reported that HLA-A*1101, HLA-B*57, and HLA-Cw*0102 contribute to the spontaneous resolution of primary HCV infection. It was reported that the DQA1*03 and DQB1*0302 alleles promote viral clearance and confer protection against chronic HCV infection in Caucasians^[20]. A number of studies have been conducted in different ethnic populations to investigate the associations of DQB1*0301 or DRB1*1101 allele with spontaneous HCV clearance^[13,21-30]. However, the results from these molecular epidemiological studies are confusing rather than being conclusive. Single study might be underpowered to detect the association between DQB1*0301 and/or DRB1*1101 allele and HCV infection because of the limited sample size. The aim of

this study was to assess the associations of HLA class II DQB1*0301 and/or DRB1*1101 allele(s) with spontaneous HCV clearance by meta-analysis of individual dataset from all studies published till date.

MATERIALS AND METHODS

Identification and eligibility of studies

PubMed was used to search for relevant reports published between 1997 and 2004 (using the key words: human leukocyte antigen, genetic polymorphism, hepatitis C virus, spontaneous clearance). The inclusion criteria for our analysis were as follows: studies reporting odds ratio (OR) or risk ratio (RR) calculated by comparing those with self-limiting infection to those with chronic infection as a measure of association; study designs such as cohort study, population-based case control study, hospital-based case-control study; studies using HLA class II molecular genotyping technique; studies including DQB1*0301 and DRB1*1101 alleles. A total of 11 candidate papers were identified.

Data extraction

Data were collected on the DQB1*0301 and DRB1*1101 allele frequencies, authors, journals, years of publication, country of origin, demographics, selection, definition of spontaneous viral clearance (anti-HCV positive and HCV-RNA negative at least for 6 mo), chronically persistent infection (anti-HCV positive and HCV-RNA positive for more than 1 year) and racial descents (categorized as Asian, European, and North American descents).

Table 2 Effect of DRB1*1101 allele on self-limiting HCV infection

Study	Spontaneous resolution <i>n</i> / <i>N</i>	Persistent infection <i>n</i> / <i>N</i>	OR (random) 95%CI
Alric	10/25	10/102	6.13 (2.18, 17.22)
Minton	11/35	11/135	5.17 (2.01, 13.27)
Cramp	9/49	6/55	1.84 (0.60, 5.60)
Mangia	7/35	24/149	1.30 (0.51, 3.32)
Thursz 1	26/85	29/170	2.14 (1.16, 3.94)
Thursz 2	14/57	15/152	2.97 (1.33, 6.65)
Fanning	4/68	4/64	0.94 (0.22, 3.92)
Alric	20/59	25/170	2.97 (1.50, 5.91)
Vejbaesya	2/43	3/57	0.88 (0.14, 5.50)
Thio	15/200	24/374	1.18 (0.61, 2.31)
Azocar	4/40	11/72	0.62 (0.18, 2.08)
Spada	2/10	2/24	2.75 (0.33, 22.92)
Total (95%CI)	124/706	164/1 524	2.02 (1.56, 2.62)

Test for heterogeneity: $\chi^2 = 19.38$, *df* = 11 ($P < 0.05$), $I^2 = 43.2\%$. Test for overall effect: $Z = 5.30$ ($P < 0.00001$).

Furthermore, we examined whether matching was used and HLA class II molecular-typing assay was validated.

Statistical analysis

The OR of sustained HCV clearance associated with HLA class II polymorphisms (DQB1*0301 and DRB1*1101 alleles) was estimated for each study. The primary analysis was to estimate the allele frequencies between the groups with viral elimination and chronic infection. For each genetic group, we estimated the heterogeneity between the studies^[31]. Data were combined using both fixed effect (Mantel-Haenszel) and random effect (DerSimonian and Laird) models^[32]. In the absence of heterogeneity, the two methods provided identical results. Random effects were more appropriate, when heterogeneity was present. Analyses were performed by the software of SAS 8.0 (SAS Institute, Cary, NC, USA) and Review Manager 4.2 (Cochrance Collaboration, Oxford, UK). All *P* values were two-sided.

RESULTS

Characteristics of the studies are shown in Table 1. Multiple studies were conducted in the European population (*n* = 9). The number of individuals with spontaneous resolution and persistent infection in DRB1*1101 and DQB1*0301 studies was 706 and 1 524, 714 and 1 497, respectively. All the subjects of these studies were selected based on a histological diagnosis of strict criteria. With two exceptions^[24,25] where the mean age of two groups differed and one where the age status was not stated^[26], all other studies were matched for age. Most studies also stated the use of matching on sex status except for two^[27,30]. Compared to other studies, only one was homogenous in terms of gender, source of HCV infection, HCV-serotype and ethnicity^[13]. This subject population was derived from a cohort of females inoculated with anti-D immunoglobulin in 1977 that was contaminated with HCV from a single source.

With regard to the anti-HCV antibody testing, most studies used a combination of two tests, while only one used a single test^[13]. Five studies used low resolution

Table 3 Effect of DQB1*0301 allele on self-limiting HCV infection

Study	Spontaneous resolution <i>n</i> / <i>N</i>	Persistent infection <i>n</i> / <i>N</i>	OR (random) 95%CI
Alric	21/25	28/91	11.81 (3.71, 37.61)
Minton	18/35	33/135	3.27 (1.51, 7.07)
Cramp	26/49	10/55	5.09 (2.10, 12.33)
Mangia	17/33	42/143	2.56 (1.18, 5.53)
Thursz 1	39/85	47/170	2.22 (1.29, 3.82)
Thursz 2	25/57	37/152	2.43 (1.28, 4.61)
Fanning	25/78	18/67	1.28 (0.63, 2.64)
Alric	38/59	45/157	4.50 (2.39, 8.50)
Vejbaesya	24/43	18/57	2.74 (1.20, 6.22)
Thio	49/200	71/374	1.38 (0.92, 2.09)
Azocar	6/40	13/72	0.80 (0.28, 2.30)
Spada	4/10	15/24	0.40 (0.09, 1.81)
Total (95%CI)	292/714	377/1 497	2.36 (1.62, 3.43)

Test for heterogeneity: $\chi^2 = 33.33$, *df* = 11 ($P = 0.0005$), $I^2 = 67.0\%$. Test for overall effect: $Z = 4.48$ ($P < 0.00001$).

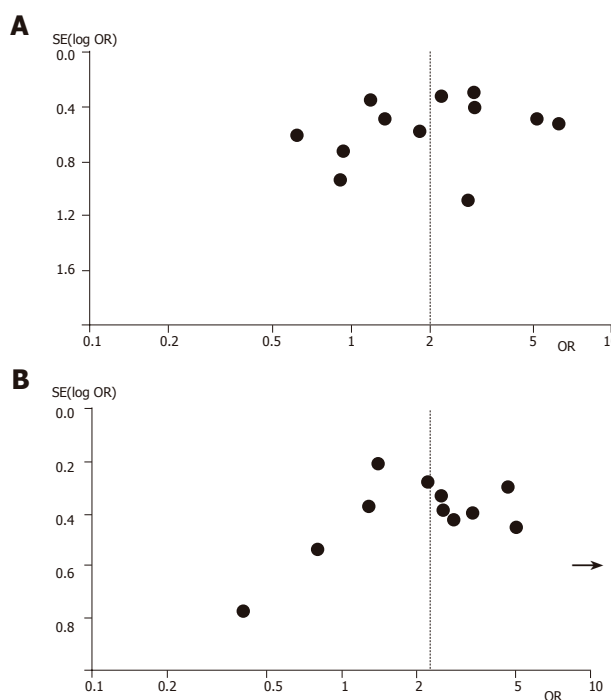


Figure 1 Funnel plot analysis of publication bias for DRB1*1101 allele (A) and DQB1*0301 allele (B). Each data point represents a separate study for the indicated association.

molecular typing (PCR-SSOP) for HLA, while others used high resolution molecular typing (PCR-SSP) for HLA^[33]. Low resolution molecular typing methods for HLA could not identify the specific alleles. Accurate methods for HLA class II typing should involve the combination of PCR-SSOP, PCR-SSP, and PCR-SSCP^[12].

Table 2 shows the OR on HCV spontaneous clearance associated with DRB1*1101 allele. Overall, individuals with DRB1*1101 allele had a significantly reduced risk for chronic hepatitis C compared to individuals without it [OR = 2.02, 95%CI (1.56, 2.62) ($P < 0.00001$)]. Similarly, subjects with DQB1*0301 allele appeared to be more

likely to clear the virus, while those lacking it were more prone to develop chronic infections [OR = 2.36, 95%CI (1.62, 3.43), $P < 0.00001$] (Table 3). These analyses were based on the data from 11 studies regardless of ethnics.

We performed tests for homogeneity with respect to the studies of DRB1*1101 and DQB1*0301 alleles and viral clearance and obtained statistically significant results (DRB1*1101: $\chi^2 = 19.38$, $P < 0.05$; DQB1*0301: $\chi^2 = 33.33$, $P = 0.0005$). Therefore, summary statistics were calculated based on a random-effect model.

Figure 1 shows the funnel plot analysis to detect publication bias of each study for DRB1*1101 and DQB1*0301, respectively. The shape of the funnel plot seemed to be asymmetrical, suggesting that publication bias might affect the findings of our meta-analysis. Furthermore, an Egger's test was used to provide statistical evidence for funnel plot symmetry^[45]. In the linear regression analysis, the intercept value provided a measure of asymmetry, the larger the deviation the more pronounced the asymmetry. The intercept value in our study was 2.33 for DQB1*0301 and 2.03 for DRB1*1101, respectively, both of them were significantly deviated from zero ($P < 0.0001$ for DQB1*0301 and $P < 0.0022$ for DRB1*1101).

DISCUSSION

DQB1*0301 allele is reproducibly involved in spontaneous viral clearance of HCV in different populations^[21-26,28,34-36]. Studies conducted in patients from France^[21], Britain^[22], Italy^[24,35] with different genetic backgrounds have identified significant genetic associations with different DQB1*0301-bearing haplotypes, suggesting that DQB1*0301 might be one of the most prominent factors in HCV clearance^[37,38].

Another allele correlated with self-limiting HCV is DRB1*1101^[21,23,25,27,34,39]. But disagreement exists over the allele. In Italy^[24,35] BRB1*1104 correlates with HCV clearance, while in Japan^[40] DR13 is linked to self-limiting infection.

It is difficult to determine whether HLA-DRB1*1101 or DQB1*0301 allele is the more relevant factor as HLA-DRB1*11 is associated with DQB1*0301. Some studies found that DRB1*1101 rather than DQB1*0301 is closely associated with viral elimination^[23,30,41,42]. While some findings suggest that DQB1*0301 is dominant in determining the outcome of HCV infection^[21,22,27,43,44]. In our meta-analysis, the OR for the two alleles was virtually identical, but which one is responsible for the viral elimination is not clear.

The outcome of HCV infection varies substantially among the individuals. Though the exact mechanisms involved in viral clearance have not been fully elucidated, there is evidence that cellular immune response, especially T-helper lymphocyte responses to HCV, plays an important role in the control of persistent HCV infection. DQB1*0301, DRB1*1101 alleles are closely associated with self-limiting HCV infection. Our meta-analysis demonstrated that subjects with HLA-DRB1*1101 allele reduced 102% risk of developing chronic HCV infection

and those carrying HLA-DQB1*0301 allele reduced 136% risk of persistent infection. HLA-DQB1*0301 and DRB1*1101 alleles might act as the most prominent factors favoring the elimination of HCV and conferring protection against chronic evolution.

There are two possible explanations for these observations. (1) Because HCV is not cytopathic for infected cells, the immune response may play an important role in HCV infection. Binding to HCV peptides of HLA molecules is the first step for the initiation of this immune response. Due to the extensive polymorphisms of HLA, the strength of the response may vary among the individuals. It is assumed that DQB1*0301 and DRB1*1101 alleles may present HCV epitopes more effectively to CD4⁺ Th cells than others, resulting in vigorous proliferative response to HCV and probably disease recovery^[37,43,46-47]. CD4⁺ Th responses have been characterized in patients with self-limiting infection and most immunodominant epitopes are present in the context of DQB1*0301 and DRB1*1101 molecules^[48]. Therefore, individuals with these two alleles may present peptides derived from HCV in a more efficient manner than those without, thus clearing the infection spontaneously. (2) Another explanation for the association between the two alleles and viral elimination may be due to the other protective HLA loci^[22,36]. Interpreting results of genetic studies can be complicated by linkage disequilibrium of the implicated gene linked to the true gene of interest^[12]. Other candidate susceptible genes located in the HLA class II region also require antigen presentation. An excess of DQB1*0301 and DRB1*1101-restricted peptide-specific responses in individuals, who clear the virus, needs to be shown in order to prove conclusively whether the mechanism of the gene effect is direct or indirect.

While there are some consistent observations on DQB1*0301 and DRB1*1101 with self-limiting infection, many results are not uniform. These inconsistencies may be due to ethnic differences, patient selection, sample size, HCV-serotype, and HLA typing technique. Firstly, a primary cause for the difference in results by different authors may be related to the great variability of the frequency of HLA alleles in different populations. It is quite possible that one ethnic group may have the preferential use of different allele(s) in viral eradication compared to other ethnic groups. This DRB1*1101 association with viral clearance was not observed in the Irish anti-D population, which might be explained by the lower frequency of DRB1*1101 in Irish^[16] (6.4%) than in French^[21] control populations (25.2%). The association of DRB1*0101 and DQB1*0501 alleles with viral clearance has been documented in Caucasian Americans, whereas in African-American patients viral clearance is mainly associated with DQB1*0301^[28]. Another study showed that there are marked differences in the frequency of viral clearance in Caucasians and African-Americans^[49]. Racial heterogeneity has to be taken into account in the future studies^[50,51]. Secondly, many studies were conducted on relatively smaller samples. Insufficient number of

individuals might decrease the power to detect a difference in the distribution of DQB1*0301 and DRB1*1101 alleles between patients with resolving infection and those with chronic evolution, though a true difference exists^[52]. Lack of an association may not mean that associations do not exist. Thirdly, a number of HLA studies concerning the association between HLA class II and outcome of infection have compared allele frequency in HCV patients to that in healthy controls^[20,53,54]. Studies including both HCV RNA positive and negative patients have failed to discover particular genetic associations^[20]. Fourthly, effects of interactions in other environmental/behavioral and/or viral factors may be inevitable. There is a clear correlation between the HLA haplotype of individuals and outcome of HCV infection^[55]. A complex interplay between various genes is likely to modulate the anti-HCV response rather than a single allele. Moreover, several variables including young age at infection and female sex have been suggested to determine HCV clearance^[17,30,56]. Another possible reason for the discrepancy between these studies lies in the viral genotype.

In conclusion, large and well-designed studies should be carried out to investigate the host genetic determinants for HCV infection. Finding an association between specific alleles and favorable clinical outcomes in HCV patients might open new avenues to explore and understand the pathogenesis of HCV.

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• RAPID COMMUNICATION •

Pyrrolidine dithiocarbamate reduces ischemia-reperfusion injury of the small intestine

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Abstract

AIM: To evaluate whether pyrrolidine dithiocarbamate (PDTC), an enhancer of HO production, attenuates intestinal IR injury.

METHODS: Eighteen male rats were randomly allocated into three groups: (a) sham; (b) IR, consisting of 30 min of intestinal ischemia, followed by 2-h period of reperfusion; and (c) PDTC treatment before IR. Intestinal microvascular perfusion (IMP) was monitored continuously by laser Doppler flowmetry. At the end of the reperfusion, serum samples for lactate dehydrogenase (LDH) levels and biopsies of ileum were obtained. HO activity in the ileum was assessed at the end of the reperfusion period.

RESULTS: At the end of the reperfusion in the IR group, IMP recovered partially to 42.5% of baseline ($P < 0.05$ vs sham), whereas PDTC improved IMP to 67.3% of baseline ($P < 0.01$ vs IR). There was a twofold increase in HO activity in PDTC group ($2.062.66 \pm 106.11$) as compared to IR (842.3 ± 85.12) ($P < 0.001$). LDH was significantly reduced ($P < 0.001$) in PDTC group (585.6 ± 102.4) as compared to IR group ($1.973.8 \pm 306.5$). Histological examination showed that the ileal mucosa was significantly less injured in PDTC group as compared with IR group.

CONCLUSION: Our study demonstrates that PDTC improves the IMP and attenuates IR injury of the intestine possibly via HO production. Additional studies are warranted to evaluate the clinical efficacy of PDTC in the prevention of IR injury of the small intestine.

INTRODUCTION

Intestinal ischemia and subsequent reperfusion (IR) injury is encountered in a variety of clinical conditions, including hypovolemic shock^[1], strangulation obstruction^[2], cardiovascular surgery^[3], abdominal aortic surgery^[4], and small bowel transplantation^[5]. The attenuation of IR injury is vital for intestinal graft function and survival after small bowel transplantation^[6]. IR injury is associated with the breakdown of microvascular perfusion with subsequent impairment of tissue oxygenation^[7]. The maintenance of microvascular perfusion is the ultimate determinant of viability of an organ^[8]. IR injury of the intestine is a systemic phenomenon resulting in bacterial translocation^[9], endotoxemia^[10], acute respiratory distress syndrome^[11], acute hepatic injury^[12], which may eventually lead to multiple organ dysfunction syndrome^[13].

Therapeutic strategies aimed at ameliorating IR injury have focused both on preventing the effects of reactive oxygen species and on downregulating the signal transduction cascades related to the expression of pro-inflammatory genes. Pharmacologic preconditioning based on enhancing the production or activity of endogenous protective molecules has also been proposed as an alternative therapeutic intervention. Among such agents, pyrrolidine dithiocarbamate (PDTC) has a variety of biochemical activities, such as redox state alternation, chelation of heavy metals, and enzyme inhibition^[14]. PDTC was initially regarded as a potent inhibitor of nuclear factor-kappa B (NF- κ B) and used as an antioxidant compound to counteract the toxic effects of free radicals^[15]. PDTC is one of the most effective inducers of heme oxygenase-1 (HO-1), which also confers cytoprotection against oxidative stress^[16]. HO is the rate-limiting enzyme in the conversion of heme into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe^{2+})^[17]. Three isoforms of HO have so far been identified: inducible HO-1; constitutively expressed HO-2; and HO-3 which is related to HO-2, but is less well characterized^[17]. The HO system is thought to

play a pivotal role in the maintenance of antioxidant and oxidant homeostasis during cellular injury. The HO system exerts four major beneficial effects broadly: (a) antioxidant function; (b) maintenance of the microcirculation; (c) anti-apoptosis; and (d) anti-inflammatory function^[17]. The antioxidant function relies on heme degradation, production of bilirubin^[18], and the formation of ferritin via Fe²⁺^[19]. The production of CO with its vasodilatory and anti-platelet properties maintains the microcirculation and may be involved in anti-apoptotic and cell arrest mechanisms. The HO system exerts anti-inflammatory effects via the modulation of endothelial adhesion molecules.

To the best of our knowledge, there are no previous studies exploring the effect of PDTC on IR injury of the intestine. Therefore, we have designed the present study to examine whether PDTC preconditioning induces HO expression in the small intestine and reduces the inflammatory response during reperfusion by focusing on the intestinal microvascular perfusion.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 250–300 g, were used in this study. Rats were kept in a temperature-controlled environment with 12-h light-dark cycle and allowed tap water and standard rat chow pellets *ad libitum*. Animal care and experimental protocols were performed in accordance with the UK Government Guidance in the Operation of the Animals (Scientific Procedures) Act 1986.

Surgical procedures

Animals were anaesthetized using isoflurane (Baxter, Norfolk, UK) and allowed to breathe spontaneously via concentric mask connected to an oxygen regulator. The animal's body temperature was maintained at 36–38 °C using a heating pad (Harvard Apparatus Ltd, Kent, UK) and monitored with a rectal temperature probe. The arterial oxygen saturation (SaO₂) and heart rate (HR) were continuously monitored with a pulse oximeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda Louisville Co., USA). The left carotid artery was cannulated with a polyethylene catheter (0.76 mm inner diameter, Portex, Kent, UK) and connected to a pressure transducer for the monitoring of mean arterial blood pressure (MABP). The right jugular vein was cannulated with a smaller polyethylene catheter (0.40 mm inner diameter, Portex, Kent, UK) for the administration of normal saline (1 mL/100 g body weight/h) to compensate for intra-operative fluid evaporation. All animals had an intravenous bolus of heparin (20 U/kg) to prevent potential thrombus formation in the ischemic segment of the intestine due to hemostasis.

Laparotomy was carried out through a midline incision. The superior mesenteric artery (SMA) was identified and dissected out from the mesentery to enable the passage of a 4/0 vicryl (Ethicon, Edinburgh, UK) suture loop. The SMA was occluded according to the method described by Arumugam *et al.*^[20]. The free ends of the vicryl suture were then passed through a 5-cm segment of polyethylene

tube (1.4 mm inner diameter). The tube was then gently advanced over the suture onto the mesentery and fixed in place with a hemostat. Reperfusion started when the hemostat was released. The animal's abdomen was covered with a plastic wrap (Saran wrap) to prevent fluid evaporation. At the end of the experiment, the animals were killed by exsanguination.

Experimental design

Rats were randomly allocated to three study groups ($n = 6$ /group). Group 1: sham laparotomy, the SMA was identified and passage of vicryl suture was performed, but without vascular occlusion. Group 2: IR, the SMA was clamped for 30 min, followed by a 2-h period of reperfusion. Group 3: PDTC+IR, the animals received intramuscularly a single dose of 100 mg/kg of PDTC (Sigma Chemical Co., St. Louis, MO, USA) 30 min before IR.

Measurement of intestinal microvascular perfusion

Intestinal microvascular perfusion was measured by a surface laser Doppler flowmeter (LDF, DRT4, Moor Instruments Limited, Axminster, UK) in flux units. The Doppler signal varies linearly with the product of the total number of moving red blood cells in the measured volume of a few cubic millimeter multiplied by the mean velocity of these red blood cells. The numeric product is termed as perfusion units or blood cell flux units. LDF has been validated as a method for measuring gastrointestinal microvascular blood flow in animal models^[21] and in clinical studies^[22]. The LDF probe was placed on a fixed site on the serosa of the ileum and was held in place by a probe holder. Serosal blood flow has previously been shown to correlate well with mucosal flow^[23].

Heme oxygenase assay

HO activity in ileal microsomal fractions was measured using a spectrophotometric assay of bilirubin production according to the method previously described by Motterlini *et al.*^[24]. Briefly, tissue microsomes were added to the following mixture: MgCl₂ (2 mmol/L), phosphate-buffered saline (100 mmol/L, pH 7.4; Sigma, UK), rat liver cytosol as a source of biliverdin reductase (3 mg of total protein), hemin (10 µmol/L; Sigma, UK), glucose-6-phosphate (2 mmol/L; Sigma, UK), glucose-6-phosphate dehydrogenase (0.2 U; Sigma, UK) and NADPH (0.8 mmol/L; Sigma, UK). The reaction was conducted in the dark for 30 min at 37 °C and terminated by the addition of chloroform (Sigma, UK). The amount of the extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm and an extinction coefficient of 40 mmol/L/cm was used for bilirubin. The total protein content of the samples was determined using a colorimetric assay according to the manufacturer's instructions (Bio-Rad, UK), and bovine gamma globulin was used as a standard.

Biochemical assays

Blood samples were taken at the end of the experiments from the carotid artery. The blood samples were

Table 1 Systemic hemodynamic parameters in three experimental groups at baseline, 5 min before, at the end of 30 min ischemia and at 1 and 2 h post-reperfusion

Hemodynamic parameters	Baseline	End of ischemia	1 h of reperfusion	2 h of reperfusion	P values
Heart rate (BPM)					
Sham	250±5	252±3	240±8	230±10	>0.05
IR	251±2	252±2	220±4	227±12	>0.05
PDTC	252±3	251±3	240±10	232±7	>0.05
SaO ₂ (%)					
Sham	98±2	95±2	94±3	95±2	>0.05
IR	98±1	95±2	97±2	95±3	>0.05
PDTC	98±1	96±3	93±1	96±3	>0.05
MABP (mmHg)					
Sham	83±6	76±5	74±6	78±8	>0.05
IR	90±3	108±5 ^b	81±3	72±5	<0.01
PDTC	87±5	107±5 ^b	79±4	73±6	<0.01

Values are expressed as mean±SE of six animals in each group. SaO₂: arterial oxygen saturation; MABP: mean arterial blood pressure. ^b*P*<0.01 vs baseline.

centrifuged at 2 000 g for 10 min at room temperature to sediment the erythrocytes. The serum was removed and analyzed on a Hitachi 747 auto-analyzer (Hitachi Ltd, Tokyo, Japan) by using commercially available enzymatic kits (Boehringer Mannheim Ltd, East Sussex, UK) for lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Histological investigation

At the end of the experiment, tissue samples of ileum were taken, fixed in 10% neutral buffered formalin and embedded in paraffin, and 4-μm-thick sections were cut using a microtome and mounted on slides for hematoxylin and eosin staining. Ileal injury was assessed using a scoring system devised by Chiu *et al.*^[25] under light microscopy without the knowledge of study groups.

Data collection and statistical analysis

Data from the pulse oximeter, pressure transducer and LDF were fed into a laptop computer and collected continuously at a sampling rate of 2 Hz. The data were calculated as 1-min averages at baseline and every 30 min till the end of the experiment. All values are expressed as mean±SE. ANOVA and Bonferroni adjustment for multiple comparisons were used unless otherwise stated, where unpaired Student's *t*-test was used for statistical analysis between groups. *P*<0.05 was considered statistically significant.

RESULTS

Systemic hemodynamic parameters

In all the animals in the experimental groups, the HR and the SaO₂ did not change significantly throughout the experiment (Table 1). In the sham group, MABP did not change significantly throughout the experiment. However, during ischemia, there was a transient increase in MABP from the baseline values in both IR (11.97±0.53 to 14.50±0.67 KPa, *P*<0.01) and PDTC groups (11.70±0.67 to 14.23±1.33 KPa, *P*<0.01). However, the MABP did not change significantly during the reperfusion period between

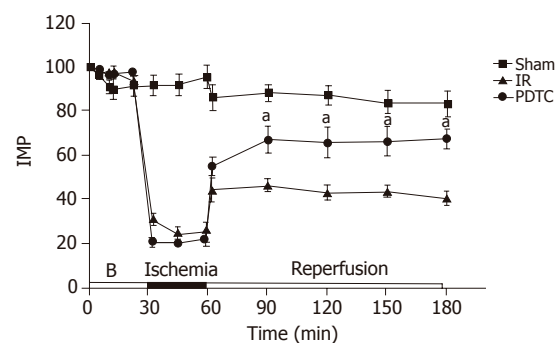


Figure 1 Intestinal microvascular perfusion in (% of baseline) during 30 min of ischemia and 2 h of reperfusion measured by L-Df. Values are expressed as mean±SE of six animals in each group (^a*P*<0.05 vs IR). B: Baseline.

the three groups (Table 1).

Intestinal microvascular perfusion

IMP did not alter significantly during the course of the experiment in the sham group. There were significant differences between IR and PDTC groups in IMP at 30 min of reperfusion (46.7±2.8% in IR vs 67.3±6.2% in PDTC, *P*<0.01). The increase in IMP persisted till the end of the 2-h reperfusion period (42.5±2.8% in IR vs 69.1±4.5% in PDTC, *P*<0.01) (Figure 1).

HO activity assay

The mean HO activity in the sham group was 409.66±62.95 pmol bilirubin/(mg protein h). PDTC led to a twofold increase in HO activity (2 085.83±158.65) as compared to IR (768.66±103.82) (*P*<0.001, Figure 2).

Biochemical analysis

LDH was significantly reduced in PDTC group (576.3±98.7) as compared to IR group (1 866.0±267.5) (*P*<0.001, Figure 3). The LDH value in sham group at the end of the reperfusion was 335.23±77.7. Significant changes were not detected in serum indicators of liver function (AST and ALT) in any of the groups (data not shown).

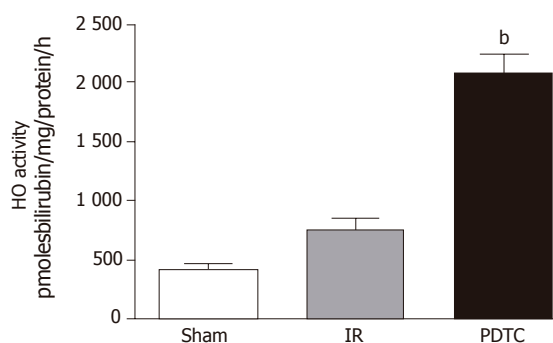


Figure 2 Ileal HO activity in all three experimental groups at the end of 2 h of reperfusion (^b $P<0.01$ vs IR).

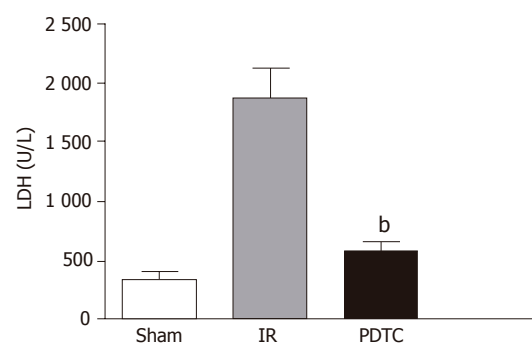


Figure 3 Serum LDH levels (U/L) at the end of 2 h reperfusion period. Values are expressed as mean \pm SE of six animals in each group. In PDTC group, LDH was significantly lower compared to the IR group (^b $P<0.001$ vs IR).

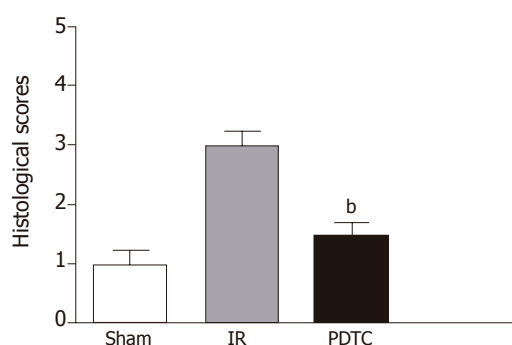


Figure 4 Comparison of histological scores of ileal mucosa between three experimental groups (^b $P<0.01$ vs IR).

Histological analysis

IR resulted in an increase in the histological score from a mean of 1.0 ± 0.2 in sham-operated animals to 3 ± 0.2 . PDTC treatment improved the histological score significantly to 1.5 ± 0.5 ($P<0.01$, Figures 4 and 5).

DISCUSSION

The present study clearly showed that PDTC improved the small bowel microvascular perfusion and increased HO activity, while ameliorating IR injury. Serum LDH levels, which is used as a marker for intestinal injury^[6], reflected intestinal damage in IR group, whereas PDTC decreased these levels. IR injury resulted in villous and crypt damage, which attributed to hemorrhage and necrosis, whereas PDTC ameliorated this effect. These results suggest that PDTC protects small bowel from IR injury possibly via HO production.

The model of 30-min period of SMA occlusion with 2-h reperfusion was reliable with no procedure-related mortality, suggesting that 30 min of intestinal ischemia is non-lethal but induces substantial intestinal injury. A considerable number of experimental studies have indicated that IR injury of the intestine occurs in a biphasic manner characterized by different time frames and mechanisms: (a) an early phase that immediately

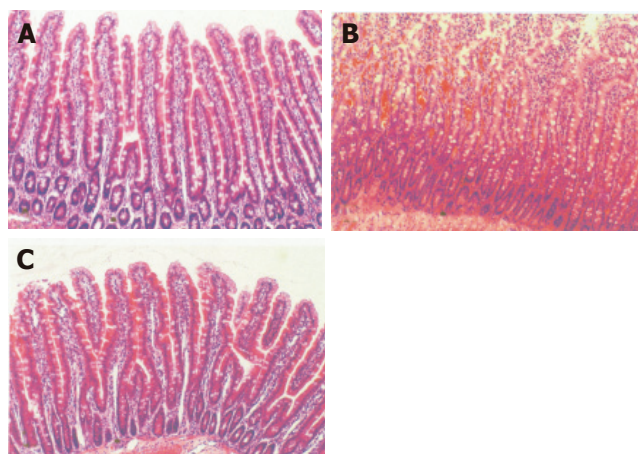


Figure 5 Representative photomicrographs of histological sections of ileum (a) in sham operated animals, (b) subjected to 30-min period of ischemia and 2-h period of reperfusion (IR) and (c) subjected to PDTC+IR (H&E, original magnification $\times 100$).

follows the ischemia and lasts for 2-3 h; and (b) a late phase which begins 12-24 h from the ischemia and lasts for about 3-4 d^[13]. Hence, a period of 2 h of reperfusion following ischemia was chosen to assess the changes in the early phase of reperfusion injury. One of the effective approaches for attenuating IR injury is to induce endogenous antioxidant genes, such as HO-1.

PDTC has been shown to be a potent inducer of HO-1 in many experimental models^[16,14]. Whereas increasing evidences suggest that HO-1 induction may mediate cellular protection against oxidant injury in both *in vitro* and *in vivo* models, the role of HO in the intestinal microcirculation is poorly understood. In this study, the effects of PDTC on IR-induced intestinal microcirculatory changes were studied by continuously measuring intestinal blood flow with LDF, which has been used extensively in both animals and human beings^[22]. It is non-invasive, safe, and easy to use^[23]. This technique allows repeated measurements of blood flow without access to venous or arterial blood. In our study, the comparison of IMP between the groups at different time points showed that PDTC increased the IMP in the initial

30 min of reperfusion, suggesting that the mechanism of pharmacologic preconditioning by PDTC modulating flow in the microcirculation is an immediate phenomenon. Therefore, the effect of PDTC is likely to involve modulation of immediate microcirculatory events at the level of capillaries and postcapillary venules, because these are the primary sites of microcirculatory failure induced by IR injury.

The results of the present study clearly demonstrated that PDTC induced maintenance of adequate microvascular perfusion *in vivo*, and indicated that this protective effect might be mediated through the action of HO, which catalyzes the conversion of heme to CO, biliverdin and free iron. CO and its action on capillary pericytes may play a major role in this protection. It is well known that capillaries are associated abluminally with pericytes which protrude primary cytoplasmic processes running along the axes of the capillaries^[26]. From these primary processes, lateral processes arise, which completely encircle the capillaries and form tight connections to the endothelial cells^[26]. These pericytes contain muscle cytoskeletal proteins, in particular alpha-smooth muscle actin, which regulate microvascular blood flow^[27]. In the liver, Ito cells, which are the sinusoids-associated pericytes, are primarily responsible for CO-mediated regulation in sinusoidal blood flow^[28]. In addition, there is an increasing evidence that CO inhibits the aggregation of platelets^[29], which could result in highly improved microvascular perfusion because of unhindered flow. Hence, tissue viability increases because of improved nutritional supply and better elimination of toxic residues of oxidative stress. Recently, Nakao *et al.*^[30] demonstrated that inhalation of CO ameliorates IR injury in a model of bowel transplantation.

Biliverdin is subjected to further degradation to bilirubin by the cytosolic enzyme biliverdin reductase^[31]. It acts as an antioxidant and is capable of scavenging oxygen free radicals that are thought to be primarily responsible for the tissue injury^[18]. Iron, the last product of heme breakdown, acts as an oxidant like other transition metals and catalyzes the formation of reactive hydroxyl radicals (OH·) by the Haber-Weiss reaction. Typically, the OH· causes biological damage by stimulating the free chain reaction known as lipid peroxidation, in which OH· attacks the fatty acid side chains of the membrane phospholipids and causes organelle and cell disruption^[32]. Therefore, it seems to be of paramount importance to eliminate free iron from the tissue in order to maintain the cellular integrity after the stress event. To enable this process, an additional expression of ferritin, the iron-binding protein, is induced simultaneously by HO^[33].

In summary, this study has demonstrated that intestinal IR injury induces rapid microcirculatory breakdown with tissue damage. Administration of PDTC maintains the intestinal microvascular blood flow and markedly attenuates the IR injury. PDTC may be of particular value in preventing IR injury to the small intestine and might help to improve the results of small bowel transplantation. Intestinal IR injury is also an obligatory component of

numerous surgical procedures. The results of the present study may, therefore, prove beneficial in many areas of clinical research. Further studies are clearly warranted to evaluate the clinical efficacy of PDTC in the prevention of IR injury of the small intestine.

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• RAPID COMMUNICATION •

Plasma leptin and ghrelin concentrations in patients with Crohn's disease

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Abstract

AIM: To determine the concentrations of leptin and ghrelin, which have opposite effects on appetite, energy expenditure, and weight control, in the plasma of patients with Crohn's disease (CD), which is often associated with weight loss and malnutrition.

METHODS: Plasma leptin and ghrelin concentrations were determined in 28 outpatients with CD by radioimmunoassay. Age- and sex-matched controls with and without *Helicobacter pylori* (*H. pylori*) infection (28 for each) were enrolled in the study. Circulating levels of these hormones were assessed with respect to CD activity, disease localization and medical treatment.

RESULTS: There were no significant differences in ghrelin levels between CD patients and *H. pylori*-negative controls. However, circulating ghrelin levels were significantly lower in *H. pylori*-infected subjects than in CD patients and uninfected controls. Plasma leptin levels were comparable among the groups. Localization and medication profile had no significant impact on circulating ghrelin and leptin levels.

CONCLUSION: Apart from *H. pylori* infection, CD itself has no significant influence on circulating ghrelin and leptin levels in the outpatients who were mostly in inactive state.

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Key words: Crohn's disease; Ghrelin; Leptin; *Helicobacter pylori*

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INTRODUCTION

Crohn's disease (CD) is characterized by a relapsing inflammatory process throughout the digestive tract^[1,2]. CD is frequently accompanied with malnutrition and weight loss^[1,2]. Possible explanations for these complications include malabsorption of nutrients, intestinal losses during inflammatory process, and anorexia^[3]. However, the exact etiology is not completely understood.

Leptin is mainly synthesized by the adipose tissue and plays a crucial role in the homeostasis of the body weight by reducing appetite and increasing energy expenditure^[4,5]. Contrary to the initial reports, leptin production is not restricted to adipocytes. It is also detected in the human placenta, muscles, and gastric chief cells^[6-10].

Ghrelin is a novel endogenous ligand for growth hormone secretagogue receptor^[11,12]. It was originally isolated from the stomach and has been subsequently identified in various tissues including the small and large intestine^[11,12]. In addition to its potent growth hormone-releasing activity, ghrelin displays metabolic effects opposed to those of leptin^[11-13]. It stimulates food intake, enhances the use of carbohydrates and reduces fat utilization. In fact, circulating ghrelin levels are decreased in obesity and increased in anorexia nervosa or cachexia^[11-14].

At present, no data on the interplay of ghrelin and leptin in CD are available. This is the first report to assess the circulating ghrelin and leptin concentrations in patients with CD simultaneously.

MATERIALS AND METHODS

Patients

Twenty-eight consecutive outpatients with CD were enrolled in the study between October 2002 and

Table 1 Plasma leptin and ghrelin levels in patients with CD and controls with or without *H pylori* infection (mean±SD)

	CD	Controls	
		<i>H pylori</i> -infected	<i>H pylori</i> -uninfected
Body mass index (kg/m ²)	20.5±0.4 (16.2-29.9) ¹	21.3±0.8 (16.4-28.5)	20.8±0.5 (16.4-26.7)
Plasma ghrelin levels (fmol/mL)	195.7±1.5 (45.7-368.7)	143.4±7.1 (53.2-481.0)	182.4±1.4 (75.7-322.2)
Plasma leptin levels (ng/mL)	3.8±0.4 (0.0-15.0)	4.3±0.6 (0.8-8.4)	3.8±0.6 (0.0-10.8)

¹Mean±SD (range).**Table 2** Plasma leptin and ghrelin levels in terms of various parameters (mean±SD)

	Ghrelin (fmol/mL)	Leptin (ng/mL)	Body mass index
Disease activity			
Active (<i>n</i> = 5)	220.6±98.8	4.7±4.1	19.9±4.5
Inactive (<i>n</i> = 23)	202.3±86.4	3.6±2.4	20.6±2.7
Disease location			
Small intestine (<i>n</i> = 3)	186.3±68.6	2.8±2.3	18.5±1.6
Large intestine (<i>n</i> = 10)	246.8±83.3	4.2±3.3	20.9±2.4
Small and large intestine (<i>n</i> = 15)	186.8±92.4	4.1±3.1	20.5±4.0
Anal lesion			
Present (<i>n</i> = 7)	213.4±85.1	3.4±2.5	20.1±4.8
Absent (<i>n</i> = 21)	206.5±92.4	4.1±3.2	20.5±2.6
Medical treatment			
5-Aminosalicylates (<i>n</i> = 16)	185.5±86.5	2.8±1.5	20.1±2.4
5-Aminosalicylates and elementary diet (<i>n</i> = 7)	229.5±98.9	4.8±3.6	20.1±5.2
No medications (<i>n</i> = 5)	243.4±76.8	5.7±4.8	21.8±3.1
<i>Helicobacter pylori</i> status			
Positive (<i>n</i> = 5)	265.8±109.8	3.5±2.6	20.2±3.4
Negative (<i>n</i> = 23)	195.7±81.5	5.5±3.9	21.8±0.1

December 2004. The study was approved by Nagasaki University Human Ethics Committee. All samples were obtained with written informed consent of the patients prior to their inclusion, in accordance with the Helsinki Declaration. A diagnosis of CD is based on the generally accepted clinical, radiographic, endoscopic and histologic criteria^[1,2]. The exclusion criteria were age <18 or >80 years, pregnancy, body mass index (BMI) >30 kg/m², diabetes mellitus, systemic infection, thyroid and liver diseases, renal impairment, use of medications against *H pylori* during the preceding 3 mo, alcohol abuse, drug addiction, and long-term corticosteroid or nonsteroidal anti-inflammatory drug use. None underwent gastrointestinal surgery. They included 16 men and 12 women, aged between 18 and 56 years (mean, 32 years). According to the CD activity index (>150), 5 patients suffered from active CD. As for disease localization, the disease process was limited to the small bowel in 3 patients, to the colon in 10 and affected both regions in 15. Seven had anal lesions and none had upper gastrointestinal involvement. Of those, 16 were treated with 5-aminosalicylates, 7 with 5-aminosalicylates and elementary diet, and 5 received no medications.

Since ghrelin is primarily produced by X/A-like cells in the gastric fundus^[11,12], its production and release may be affected by inflammatory and atrophic events associated with *H pylori* infection^[15,16]. Thus, *H pylori* status was assessed, as described below.

Age- and sex-matched 23 *H pylori*-negative healthy subjects served as controls. Moreover, age- and sex-

matched 23 *H pylori*-infected patients with non-ulcer dyspepsia were enrolled in the study.

Plasma leptin and ghrelin concentrations

Blood samples were taken between 9 and 11 a.m. after an overnight fast, transferred into chilled tubes containing ethylenediaminetetraacetic acid-2Na and aprotinin, stored on ice during collection and centrifuged. Then, the plasma was separated, and stored at -80 °C until assay. Plasma ghrelin concentrations were measured in-house in duplicate by radioimmunoassay (RIA), as described previously^[17]. This assay system employs a rabbit polyclonal antibody against the C-terminal fragment of human ghrelin. Plasma leptin concentrations were measured in duplicate by commercial RIA kit (Linco Research Co., St. Charles, USA), based on the protocol provided by the manufacturer.

Detection of *H pylori* infection

H pylori status was assessed by anti-*H pylori* immunoglobulin G antibody (HELp TEST, an enzyme linked immunosorbent assay kit, AMRAD Co., Melbourne, Australia) using the stored plasma and ¹³C-urea breath test (UBiT, Otsuka Pharmaceutical Co., Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using Fisher's exact, χ^2 , Student's *t*, Mann-Whitney *U*, Kruskal-Wallis, Spearman's rank, and Wilcoxon signed ranks tests, as appropriate. *P*<0.05 was considered statistically significant. Data were expressed as mean±SD.

RESULTS

There were no significant differences in ghrelin levels between CD patients and *H pylori*-negative controls (Table 1). However, circulating ghrelin levels were significantly lower in *H pylori*-infected subjects than in CD patients ($P < 0.01$, Table 1) and controls negative for the infection ($P < 0.05$, Table 1). On the other hand, circulating leptin levels were comparable between the groups (Table 1).

Other parameters such as disease activity, localization and medication profile and *H pylori* status had no significant impact on circulating ghrelin and leptin levels (Table 2).

There was a significant positive correlation between plasma leptin levels and BMI ($r = 0.61$, $P < 0.005$). Plasma ghrelin concentrations tended to decrease with increase in BMI, *albeit* insignificantly. There was no significant correlation between circulating ghrelin and leptin levels.

DISCUSSION

Our results suggest that circulating ghrelin levels are not altered in CD patients who mainly consisted of those with the inactive disease. In our study, plasma concentrations of leptin, the opposing metabolic counterpart of ghrelin^[10-13], were not affected by the disease. In addition, there were no significant association of such factors as localization and medication profile with circulating ghrelin and leptin. These findings suggest that alterations in these hormones involved in appetite and energy metabolism are unlikely to mediate nutrition state in CD. However, these results must be interpreted within the context of the limitations in our study. First, the sample size was relatively small. Second, severe patients with wasting symptoms or malnutrition were not enrolled in this study, as it was in the outpatient-based setting. Although our series were not associated with upper gastrointestinal lesions, where ghrelin is primarily produced^[10,17], such involvement might have an impact on the circulating ghrelin levels.

Murch *et al.*^[18] demonstrated that suppression of growth velocity in children with CD correlates with circulating tumor necrosis factor (TNF) α concentrations. In cachectic state, a positive correlation has been found between ghrelin and TNF α circulating levels^[19]. On the other hand, there is a significant association between serum levels of leptin and TNF receptor 1^[20]. We did not measure TNF α and TNF receptor 1 levels in the present series of CD, but accumulating evidence indicates that the TNF system is activated in CD^[1,2].

Recently, Suzuki *et al.*^[15] demonstrated that *H pylori* infection modifies gastric and plasma ghrelin dynamics in Mongolian gerbils. There are contradictory reports on the relationship between *H pylori* and ghrelin. A Turkish study reported that *H pylori* has no effect on plasma ghrelin levels^[21], whereas a British study demonstrated that circulating ghrelin increases following the cure of *H pylori* infection^[16]. In our series, the principal determinant of circulating ghrelin might be the *H pylori*

status. The exact reason for such a discrepancy is not clear, but the following factors should be considered: differences in the study populations of diverse races, nutrient status and dietary habits, small sample size and inadequate assessment of *H pylori* status, i.e., only by histology, leading to underestimation of infection in their series^[21]. In turn, circulating leptin concentrations are not associated with *H pylori* status, consistent with previous reports^[22,23]. On the other hand, plasma leptin concentrations significantly correlate with BMI, as the primary contributor of circulating leptin is exclusively the adipose tissue^[4,5].

In conclusion, CD itself has no significant influence on the circulating levels of leptin and ghrelin. Further evaluation of a larger population with the active disease or response to medical treatment including parenteral and enteric nutrition and infliximab is warranted. Plasma ghrelin dynamics may be affected by *H pylori* status in human beings.

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• RAPID COMMUNICATION •

Accuracy of a predictive model for severe hepatic fibrosis or cirrhosis in chronic hepatitis C

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Abstract

AIM: To assess the accuracy of a model in diagnosing severe fibrosis/cirrhosis in chronic hepatitis C virus (HCV) infection.

METHODS: The model, based on the sequential combination of the Bonacini score (BS: ALT/AST ratio, platelet count and INR) and ultrasonography liver surface characteristics, was applied to 176 patients with chronic HCV infection. Assuming a pre-test probability of 35%, the model defined four levels of post-test probability of severe fibrosis/cirrhosis: <10% (low), 10-74% (not diagnostic), 75-90% (high) and >90% (almost absolute). The predicted probabilities were compared with the observed patients' distribution according to the histology (METAVIR).

RESULTS: Severe fibrosis/cirrhosis was found in 67 patients (38%). The model discriminated patients in three comparable groups: 34% with a very high (>90%) or low (<10%) probability of severe fibrosis, 33% with a probability ranging from 75% to 90%, and 33% with an uncertain diagnosis (i.e., a probability ranging from 10% to 74%). The observed frequency of severe fibrosis/cirrhosis was within the predefined ranges.

CONCLUSION: The model can correctly identify 67% of patients with a high (>75%) or low (<10%) probability of cirrhosis, leaving only 33% of the patients still requiring liver biopsy.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide: nearly 80% of HCV-infected patients develop chronic infection, and about 20% progress to cirrhosis^[1]. Liver histology is currently considered as the "reference standard" for evaluating hepatic damage on the basis of the degree of necrotic inflammatory activity and fibrosis, with the latter having prognostic significance and playing a major role in therapeutic decision making^[2,3].

However, liver biopsy is an invasive procedure with mild and severe complications of 20% and 0.5%, respectively^[4,5]. Furthermore, its sensitivity in diagnosing liver cirrhosis is not absolute and the rate of false negative results is approximately 30%^[6]. In addition, as it has been well evidenced by the recent studies, an adequate liver specimen should be at least 2.5 cm long^[7] and 1.4 mm wide^[8] including at least 6-8 portal tracts^[9]. This has recently led various groups to investigate the non-invasive methods of detecting severe fibrosis/cirrhosis, including only biochemical (ALT, AST, GGT) tests^[10-14] or in combination with hematological (platelet count) tests^[15], test panels^[16-25], serum "markers" of fibrosis (such as hyaluronic acid or procollagen peptides)^[26-30] and ultrasonographic parameters (e.g., liver surface nodularity (LSN), portal blood flow)^[31-34], but none of which have proved to be capable of avoiding liver biopsy.

The aim of this prospective study was to evaluate the accuracy of a model based on the sequential combination of a set of simple biochemical tests (Bonacini score, BS)^[19,20] and liver surface ultrasound (US) examination^[32] in diagnosing severe fibrosis/cirrhosis in a large series of consecutive patients with chronic HCV infection undergoing liver biopsy.

MATERIALS AND METHODS

All anti-HCV positive patients (EIA III, Abbott Laboratories, Chicago, IL, USA) with detectable HCV-RNA serum levels (Amplicor HCV kit, Roche, Molecular Systems, Basel, Switzerland) were evaluated for enrollment between September 2001 and June 2004.

The presence of decompensated liver disease (i.e., portosystemic encephalopathy, jaundice or ascites detected by means of US) and/or an absolute contraindication to liver biopsy (PLT <60 000/mm³, PT <60%) excluded patients from the study, whereas patients with an increase of ≥ 1.5 UNL in the serum alanine-aminotransferase (ALT) levels recorded twice in the previous 6 months, were included in the study, whose protocol was approved by the pertinent ethics committee after having obtained their written informed consent. Socio-demographic and clinical data were recorded as those on past and/or current alcohol intake for which a semi-quantitative questionnaire (<30, 30-80 and >80 g) was used. At the time of liver biopsy, the laboratory data included AST, ALT (IU/L r.v. <40), platelet count (10⁶/μL r.v. 130 000-400 000), the international normalized ratio (INR 0.8-1.2), level of albumin (g/dL r.v. 3.5-5.0), total bilirubin (mg/dL r.v. 0.3-1.0) and hemoglobin (g/dL r.v. 14-18 for men and 12-16 for women), as well as HCV genotyping by means of restriction fragment length polymorphism after amplification of the 5' non-coding region of the HCV genome^[35]. As detailed in Table 1, the "cirrhosis discriminant score" (range 0-11) of each patient was calculated according to the method of Bonacini *et al.*^[19], based on ALT/AST ratio, platelet count and INR. After an overnight fast, a US liver scan was performed using an ATL HDI 5000 equipment (Advanced Technology Laboratories, Bothell, WA, USA) and both 3.5 and a 5-12 MHz transducer by one of three gastroenterologists (AC, SM or MA) blinded to the clinical, biochemical and histologic data. A 5-12 MHz transducer was used to obtain multiple scans of the outer 2-3 cm of the liver parenchyma and of both lobes. LSN was considered positive when the liver surface appeared as a dotted or an irregular line and/or the liver parenchyma was not homogeneous, with areas of different echogenicity, reflecting an underlying nodularity. The interobserver agreement for this sign was calculated according to K statistics.

A US-guided transcostal or subcostal liver biopsy was performed (by AC or MF) using an 18-gauge needle (Biomol Hospital Service, Pomezia, Rome, Italy). We considered acceptable only specimens ≥ 2.0 cm long including >12 portal tracts, that were fixed in formalin and stained with hematoxylin-eosin, silver impregnation for reticulin, and Masson's trichrome or picosirius red for collagen. In case of inadequacy of the sample, a second biopsy was obtained.

The specimens evaluated by the same pathologist (VM) unaware of the patients' characteristics were staged according to METAVIR scoring system^[36], where F0 indicates the lack of fibrosis, F1 corresponds to portal

Table 1 Determinants of Bonacini score

Laboratory	Score						
parameters	0	1	2	3	4	5	6
INR	<1.1	1.1-1.4	>1.4				
ALT/AST ratio	>1.7	1.7-1.2	1.19-0.6	<0.6			
PLT $\times 1000/\text{mm}^3$	>340	340-280	279-220	219-160	159-100	99-40	<40

fibrosis without septa, F2 to the presence of few septa, F3 and F4 to the finding of numerous septa without or with cirrhosis, respectively. Histologic findings were considered as the reference standard for the presence and degree of fibrosis.

Statistical analysis

The pre-test probability of severe fibrosis/cirrhosis (i.e., a staging score 3-4) was estimated to be about 35% on the basis of recent data from comparable series^[2,32,37-39]. A predictive model was obtained by the sequential application of the BS and US liver surface examination, which could obtain the post-test probability of severe fibrosis/cirrhosis. As the BS and US signs of LSN could be considered conditionally independent, the probability of severe fibrosis/cirrhosis obtained by calculating the BS has been considered as the pre-test probability before the US examination. The post-test probabilities were calculated on the basis of post-test odds as previously described^[40,41]. The values of sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR-) of both BS and US in diagnosing severe fibrosis have been previously defined^[19,32].

In detail, three possibilities had to be taken into account. Firstly, for a staging score 0-2, a BS ≤ 3 had a sensitivity of 58% and a specificity of 85%, accounting for a LR+ of 4. Accordingly, in our model in the presence of a BS ≤ 3 , the probability of stage 0-2 increased from a pre-test one of 65% to 88%. Secondly, the sensitivity and specificity for severe fibrosis in the presence of a BS of 4-7 were 50%, making it impossible to modify the 35% pre-test probability of severe fibrosis/cirrhosis on the basis of a LR+ and a LR- of about one. Thirdly, the specificity of 100% for a BS >7 led to an almost absolute post-test probability of staging score 3-4, with a LR+ higher than 100. As far as the US finding of LSN was concerned, an inter-observer variability of 0.80 was reported. Our own previous data^[32] demonstrated a sensitivity of 54% and a specificity of 95% for a staging score 3-4, with a LR+ and a LR- of 11.6 and 0.5, respectively. By combining the BS and the US signs of LSN, five groups of patients (I-V) were defined (Figure 1) with different post-test probability of severe fibrosis/cirrhosis. In the last group, with BS ≥ 7 the post-test probability of a staging score of 3-4 was close to 100% regardless of the US results. Finally, as detailed in Figure 1, to test the predictive accuracy and clinical usefulness of the model, four *clinically acceptable* ranges of post-test probability of severe fibrosis/cirrhosis were predefined based on BS and US findings: *low probability* (<10%), *not diagnostic* (10-74%), *high probability* (75-90%) and *almost absolute probability* (>90%).

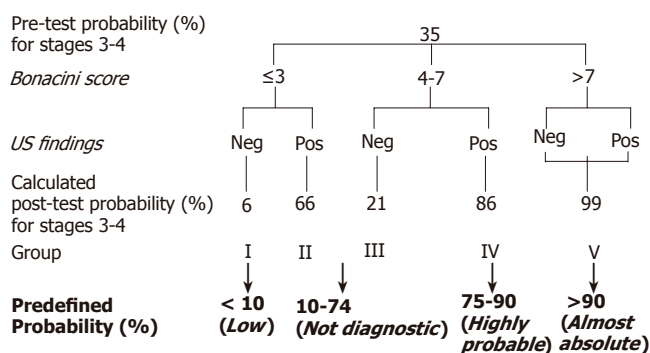


Figure 1 Post-test probability of severe fibrosis/cirrhosis calculated on the basis of both BS and US findings (starting from the pre-test value of 35%), and ranges of post-test probability for severe fibrosis/cirrhosis predefined as "clinically relevant" (in bold).

Table 2 Clinical and biochemical characteristics of 176 consecutive patients with chronic hepatitis C included in

	#	%	Mean±SD
Age (yr)			53.5±21.2
Sex: Male	96	55	
Female	80	45	
BMI (kg/m ²)			25.9±2.9
<25	90	51	
≥25	86	49	
Alcohol intake (g/d)			
≤30	142	80	
31-80	24	14	
>80	10	6	
HCV genotype			
1	92	52	
2	60	34	
3	19	11	
4	5	3	
Platelet count			181±128
AST (IU/L)			78.5±101
ALT (IU/L)			125±178
AST/ALT			1.6±1.1
INR			1.05±0.1
Hemoglobin (g/dL)			14.5±2.1
Albumin (g/dL)			4.5±0.6
Bilirubin (mg/dL)			0.9±0.2

RESULTS

During the recruitment period, 1 089 patients were investigated for liver disease, and 363 were chronically infected with HCV. Of them, 184 were excluded for the refusal of liver biopsy (#11) because of already established cirrhosis with Child-Pugh score >5 (#67), or because of normal or slightly elevated ALT levels (<1.5 times the upper normal limit) or uncompleted ALT data (less than six distinct determinations) (#106). The remaining 179 fulfilled the inclusion criteria. Based on the inadequate US findings, three were excluded accounting for an overall recruitment of 176 patients, whose characteristics are detailed in Table 2. The inter-observer agreement for the detection of the LSN sign was 0.77. The average length of liver specimens was 4.1±0.4 cm and in 18 cases a second liver sample was obtained because of the inadequacy of the first one. At liver biopsy, well tolerated in all the cases, 67 patients (38%) had severe fibrosis (#52) or

Table 3 Distribution of 176 patients according to Bonacini score and pre-defined grouping

Bonacini score	≤3	4-7	>7
LSN	-ve +ve	-ve +ve	-ve +ve
Patients (#)	56 8	49 59	0 4
Pre-defined group ¹	I II	III IV	V
F 3-4	1/56	14/49	0
Patients (#)	4/8	44/59	4/4
Observed (%)	2	29	100
Expected ² (%)	50 6 66	76 21 86	99

¹See text and Figure 1; ²As from Table 1.

cirrhosis (#15, all in Child's A class). Of the remaining 109 (62%), fibrosis was absent in 6, mild (score 1) in 40 and moderate (score 2) in 63. LSN was present in 9 out of the 109 (8%) patients with a fibrosis score of 0-2 and in 40 out of 67 (60%) with F 3-4 with a sensitivity, specificity, LR+ and LR- of 60%, 92% and 7.5 and 0.5, respectively. Patients' distribution according to the BS and LSN, and corresponding observed frequency (%) of severe fibrosis/cirrhosis (stage 3-4) *vs* expected post-test probability calculated by the model, are shown in Table 3. Overall, the diagnostic accuracy of the model was 86.5% in groups I, IV, and V, accounting for 67% of the patients. Sixteen of one hundred and nineteen patients were incorrectly defined (1 in group I with fibrosis stage ≥3, 15 in group IV with fibrosis stage <3). In groups II and III (33% of the total), the model did not show any predictive role and did not significantly modify the pre-test probability of severe fibrosis/cirrhosis.

DISCUSSION

In this prospective study, we have evaluated the accuracy of a non-invasive predictive model, consisting of the sequential combination of laboratory data score (BS) and US finding of a nodular liver surface, in detecting severe liver fibrosis or cirrhosis in patients with chronic hepatitis C. In clinical practice, it is easy to use the two elements with their operative characteristics individually validated in previous studies^[19,20,32]. The BS is based on simple and widely available laboratory tests (ALT, AST, INR and platelet count) that assure its repeatability, and LSN can be easily detected using commercially available US equipment. Previous series using platelet count or ALT and AST level or BS or US can precisely predict the presence or absence of fibrosis but with a low discriminating power; thus, few patients have a very low or high probability of fibrosis, and most of them are in the gray zone of uncertainty^[19,20,32]. On the contrary, the sequential model, which is applicable to the vast majority of enrolled patients (98%, because the US examination was technically inadequate in three cases), has been proved to have an adequate discriminating power and an accurate calibration. Its discriminating power is demonstrated by the fact that we were able to divide the patients into three comparable groups: 34% with a very

high (>90%) or low (<10%) probability of severe fibrosis, 33% with a probability ranging between 75% and 90%, and 33% with an uncertain diagnosis (i.e., with a fibrosis score 3-4 probability between 10% and 74%). The precise calibration of the model is demonstrated by the fact that the observed frequency of severe fibrosis/cirrhosis was within the predefined ranges for each group (Table 3). The validity of the model is further supported by the finding that the 38% frequency of severe fibrosis/cirrhosis in the study population was similar to the theoretical value of 35% assumed by considering series of patients with comparable characteristics, represented by a ≥ 1.5 times increase in ALT levels recorded twice during a period of 6 months or more^[2,32,37-39].

The operative characteristics of US are slightly different from those indicated by our own recent data^[32], which were used for the model. However, the variations in LR+ and LR- remained within the broad confidence intervals of the original estimates and did not critically challenge the calibration of the model. Further studies are needed to assess the generalizability of the model, i.e. its reproducibility and transportability, for which independent validations are advisable.

Although our simple model could detect the presence or absence of severe fibrosis in nearly two-thirds of the patients, thus avoiding the need for liver biopsy, some limitations of the study require further discussion. Because of its intrinsic characteristics, the model was unable to detect moderate (stage 2) fibrosis, which together with grading is relevant in therapeutic decision making. Furthermore, the study seemed to underestimate the diagnostic role of liver biopsy, which was used not only to define staging and grading, but also to detect concomitant pathologies. However, the latter role which is already questionable in patients with liver disease of uncertain etiology^[42] was irrelevant in our series of patients with chronic HCV infection. Conversely, liver biopsy still has some limitations in clinical practice, such as the difficulty in obtaining a liver sample of adequate size, as recently reported by some studies^[7,8], and which can be considered as representative of the whole liver, given the patchy distribution of liver fibrosis in chronic hepatitis C. The above limitations in our opinion are a major issue in evaluating the diagnostic accuracy of non invasive diagnostic tests compared to liver biopsy. In fact in these types of studies, the major drawback is the imperfection of the reference standard itself, which can significantly affect the estimates of the operative characteristics of the test under investigation. To reduce the possible sample size effect, in the present study we obtained all the liver specimens >3 cm with >12 portal tracts from patients.

Previous studies assessing the individual role of BS and US findings of LSN in detecting severe fibrosis have found that they are inaccurate because their low power of discrimination means that a large proportion of patients still require liver biopsy^[19,20,32]. It has recently been shown that the use of laboratory tests^[10-16] and specific fibrosis markers, such as hyaluronic acid^[26-30], is highly accurate in diagnosing cirrhosis. However, the considerable

differences in the study design and the particularly high prevalence of symptomatic cirrhosis sometimes make it difficult to compare these data to our own. It has also recently been shown that relatively complex laboratory scores are accurate in discriminating stage 0-1 from stage 2-4, but fail to confirm severe fibrosis^[17-25] although the aim of these studies was to detect patients with minimal or absent fibrosis with stage 3-4 found in only 15% (*vs* 37% in our series), possibly because they included patients with normal ALT levels and a younger age.

Recently, preliminary data from patients with chronic HCV liver disease^[43] suggest a promising role of fibroscan [one-dimensional (1-D) transient elastography] in predicting the degree of liver fibrosis with a good accuracy in discriminating stage 0-2 from 3-4, and a LR+ and LR- of 5.7 and 0.16, respectively, similar to those observed in the present series for the US sign of LSN (LR+ and LR- of 7.5 and 0.5, respectively).

In conclusion, our data suggest that the use of liver biopsy to detect severe fibrosis can be avoided in about two-thirds of patients. Furthermore, the model can also be used in patients aged more than 65 years for whom liver biopsy could be questioned.

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• RAPID COMMUNICATION •

Combined carriership of *TLR9* -1237C and *CD14* -260T alleles enhances the risk of developing chronic relapsing pouchitis

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CONCLUSION: There is no evidence that the SNPs predispose to the need for IPAA surgery. The significant increase of the combined carriership of the *CD14* -260T and *TLR9* -1237C alleles in the chronic relapsing pouchitis group suggests that these markers identify a subgroup of IPAA patients with a risk of developing chronic or refractory pouchitis.

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Key words: Pouchitis; Innate immunity; Single nucleotide polymorphisms; *CD14*; *TLR9*

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Abstract

AIM: To investigate the single nucleotide polymorphisms (SNPs) in genes involved in bacterial recognition and the susceptibility to pouchitis or pouchitis severity.

METHODS: Analyses of *CD14* -260C>T, *CARD15/NOD2* 3020insC, Toll-like receptor (*TLR*)4 +896A>G, *TLR9* -1237T>C, *TLR9*+2848G>A, and *IRAKM* +22148G>A SNPs were performed in 157 ileal-pouch anal anastomosis (IPAA) patients (79 patients who did not develop pouchitis, 43 infrequent pouchitis patients, 35 chronic relapsing pouchitis patients) and 224 Italian Caucasian healthy controls.

RESULTS: No significant differences were found in SNP frequencies between controls and IPAA patients. However, a significant difference in carriership frequency of the *TLR9*-1237C allele was found between the infrequent pouchitis and chronic relapsing pouchitis groups [$P = 0.028$, odd's ratio (OR) = 3.2, 95%CI = 1.2-8.6]. This allele uniquely represented a 4-locus *TLR9* haplotype comprising both studied *TLR9* SNPs in Caucasians. Carrier trait analysis revealed an enhanced combined carriership of the alleles *TLR9* -1237C and *CD14* -260T in the chronic relapsing pouchitis and infrequent pouchitis group ($P = 0.018$, OR = 4.1, 95%CI = 1.4 -12.3).

INTRODUCTION

Patients with ulcerative colitis may need surgery for their disease and proctocolectomy with ileal-pouch anal anastomosis (IPAA) is the surgical procedure of choice for the management of these patients^[1,2]. Most patients undergoing IPAA for severe colitis or chronic continuous disease achieve good functional results, but some patients develop pouchitis, a non-specific idiopathic inflammation of the ileal reservoir. Frequency rates of pouchitis are highly variable, ranging 10-59% depending on the length of follow-up and the diagnostic criteria used^[3]. Though the origin of pouchitis remains unknown, genetic and immunological factors are likely to be involved in addition to ileal mucosa that needs to adapt to its new role as a reservoir^[4]. This is illustrated by the fact that pouchitis occurs almost exclusively in patients with IPAA for ulcerative colitis and not in patients with IPAA for familial adenomatous polyposis, a hereditary non-inflammatory disease of the colon with high risk of developing colon cancer. An important role of luminal bacteria in the development of pouchitis is underscored by various reports on bacterial overgrowth and dysbiosis in pouchitis^[5] and is further confirmed by the efficacy of antibiotic and probiotic therapy^[6,7].

Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), are essential components of the innate

immune system as recognition of microbial products occurs via PRRs that are expressed by innate effector cells. Microbial recognition results in a rapid and efficient immune response against invading microorganisms^[8].

Given the role of luminal bacteria in driving the inflammatory response in pouchitis, identification and functional characterization of polymorphisms in innate immunity genes may provide insight in a possible genetically determined susceptibility to pouchitis and/or chronic relapsing pouchitis^[9].

CD14 is part of the endotoxin/lipopolysaccharide (LPS) receptor complex^[8] and is important in conjunction with TLR4 and with TLR2^[10] in the recognition of LPS, a membrane glycolipid on Gram-negative bacteria. CD14 may also recognize cell membrane components of Gram-positive mycobacteria and viruses^[11-15]. CD14 exists in a membrane form on monocytes and neutrophils and in a soluble form in serum^[16-18]. The SNP at position -260C>T (also known as *CD14*-159C>T) in the promoter region of the *CD14* gene (located on chromosome 5q31) is associated with enhanced transcriptional activity^[19] and significantly higher CD14 serum levels^[20]. Increased expression of CD14 in macrophages has been found in inflammatory bowel disease (IBD)^[21]. An association of the *CD14*-260C>T gene polymorphism with IBD^[22,23] and atherosclerosis^[24] has been described. Genetically determined variation in CD14 serum levels may have functional consequences given the ability of soluble CD14 to confer pathogen responsiveness to cells such as intestinal, epithelial and endothelial cells that do not express CD14 on their membranes^[25].

The *TLR4* gene is located on chromosome 9q32-q33. The *TLR4*+896A>G SNP affects the leucine-rich repeat domain of TLR4 and is associated with hyporesponsiveness to LPS^[26] with increased susceptibility to severe bacterial infections and IBD^[27] and may predispose to septic shock with Gram-negative microorganisms^[28,29].

TLR9 is required for the recognition of CpG motifs, short sequences of unmethylated DNA predominantly present in bacterial DNA. CpG motifs have immunostimulatory activity by inducing dendritic cell maturation, B-cell proliferation and production of cytokines, including interleukin-6 (IL-6) and interleukin-12 (IL-12)^[30,31]. TLR9 signaling has been shown to mediate the resolution of intestinal inflammation in experimental colitis^[32], suggesting that the release of bacterial DNA from the microflora might favor immune homeostasis. The promoter *TLR9*-1237T>C SNP located on chromosome 3p21.3 is associated with susceptibility to asthma in European Americans, but not in Hispanic or African Americans^[33] and the marker D3S1076 in this region shows association with IBD in a classical TDT test^[42]. Török and colleagues^[34] studied the *TLR9*-1237T>C and *TLR9*+2848 G>A SNPs in German patients with Crohn's disease, ulcerative colitis and healthy blood controls and found that the allele *TLR9*-1237C carrier status is associated with Crohn's disease compared to controls. *TLR9*+2848 G>A allele frequencies are not different between the study groups.

CARD15/NOD2 is a cytoplasmatic bacterial PRR

expressed in monocytes and intestinal epithelial cells and mediates response against muramyl dipeptide derived from peptidoglycan^[35,36]. The *CARD15/NOD2* gene is located on chromosome 16q12. Three major polymorphisms in this gene (R702W, G908R, and L1007 frameshift mutation) are associated with susceptibility to Crohn's disease, possibly as a result of a defective response against muramyl dipeptide derived from peptidoglycan^[37]. Recently, an increased frequency of the L1007 frameshift mutation has been observed in Italian patients with ulcerative colitis when compared to controls^[38].

The intracellular domains of TLRs are homologous to the interleukin-1 receptor (IL-1R) type I intracellular domain and use a common pathway of intracellular signaling with shared components including the protein kinase IL-1R-associated kinase1 (IRAK1) and IRAK-M, as a negative regulator of TLR signaling. Cytokine production increases in IRAK-M-/- macrophages after TLR/IL-1 stimulation and bacterial challenge, while endotoxin tolerance reduces in these cells. Furthermore, IRAK-M-/- mice have increased inflammatory responses to bacterial infection and develop intestinal inflammation. These data suggest that IRAK-M has a regulatory function in TLR/IL-1R signaling and innate immune homeostasis^[39]. The *IRAKM* (or *IRAK3*) gene is located at chromosome 12q14.2 within the IBD2 region associated with ulcerative colitis^[40,41]. Genetic variation in the *IRAKM* gene may be involved in the development of chronic intestinal inflammation. For this reason, we chose to analyze a non-synonymous SNP in exon 5 resulting in an Ile/Val substitution.

A candidate gene approach, based on the determination of frequencies of functional SNPs, can be used to investigate the relevance of genes to the disease susceptibility and severity. Carrier trait analysis investigates combinations of SNPs and allows studying the implication of different SNPs in disease susceptibility and severity as a result of their synergistic action.

The aim of this study was whether SNPs in innate immunity genes could contribute to the susceptibility to pouchitis and/or severity of pouchitis. We chose candidate genes of *CD14*, *TLR4*, *TLR9*, *NOD2/CARD15*, and *IRAKM* for their involvement in bacterial recognition and intracellular signaling pathways.

MATERIALS AND METHODS

Patients

One hundred and fifty-seven unrelated patients with IPAA for ulcerative colitis and 224 healthy blood donors were studied. All individuals were Italian Caucasians. Consent was obtained and the local ethics committee approved the protocol. Their demographic and clinical information is described in Table 1. IPAA patients were subdivided into three test groups according to the pouchitis pattern. One group consisted of IPAA patients who had never developed pouchitis, another group consisted of IPAA patients who had up to two episodes of pouchitis during IPAA (infrequent pouchitis) and third group consisted of IPAA patients who developed three or more episodes of

pouchitis (chronic relapsing pouchitis).

DNA isolation

Venous blood (5–10 mL) was drawn and genomic DNA was isolated using standard protocols. Polymorphisms of the *CD14*, *TLR4*, *TLR9*, *CARD15/NOD2*, and *IRAKM* genes in these three groups were analyzed. Genomic DNA (5–100 ng) was used for each genotyping.

Analysis of gene polymorphisms

Polymerase chain reaction (PCR) for RFLP analyses was performed on a thermal cycler GeneAmp 9700 (Perkin-Elmer Cetus, Norwalk, CT, USA). Digested fragments were analyzed on a 4% agarose gel except for the *IRAKM* SNP analyzed on a 2% agarose gel and visualized with an UV-illuminator after ethidium bromide staining. SNPs were analyzed with the TaqMan assay (Applied Biosystems, Foster City, CA, USA). MGB TaqMan probes and primer pairs were designed with Primer Express software (version 2.0). TaqMan thermocycling consisted of an initial step at 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. We used the ABI Prism 7000 sequence detector (Applied Biosystems) for data acquisition.

Genotyping

CD14-260C>T genotyping (NCBI SNP CLUSTER ID: rs2569190) was performed by PCR. Primers used were: forward primer 5'-TCA CCT CCC CAC CTC TCT T-3' and reverse primer 5'-CCT GCA GAA TCC TTC CTG TT-3'. The PCR conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min followed by cooling to 4 °C. The 107-bp amplicons were digested overnight with *HaeIII* (New England Biolabs, UK). Digestion resulted in two fragments of 83 and 24 bp (C allele) or 107 bp (T allele), respectively.

Genotyping of the *TLR4*+896 A>G SNP (NCBI SNP CLUSTER ID: rs4986790) was performed with forward primer 5'-TTT ACC CTT TCA ATA GTC ACA CTC A-3' and reverse primer 5'-AGC ATA CTT AGA CTA CTA CCT CCA TG-3'. PCR conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min followed by cooling to 4 °C. The 102-bp amplicons were digested overnight with *NcoI* (New England Biolabs, UK). Digestion resulted in two fragments of 80 and 22 bp (G allele) or 102 bp (A allele), respectively.

CARD15/NOD2 3020InsC (*CARD15* L1007G) (NCBI SNP CLUSTER ID: rs2066847) genotyping was performed with forward primer 5'-GGC AGA AGC CCT CCT GCA GGG CC-3' and reverse primer 5'-CCT CAA AAT TCT GCC ATT CC-3'. PCR conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min followed

Table 1 Demographic features of IPAA patients and healthy controls

	IPAA patients	Healthy controls
Total number (n)	157	224
Gender M/F	88/69	118/106
Mean age in yr (SD)	42.9 (11.8)	45.8 (12.8)
Range	17–73	21–77
Median	41	45.5

by cooling to 4 °C. The 150-bp amplicons were digested overnight with *ApaI*. Digestion resulted in a fragment of 150 bp (no insertion) or 128 bp and 22 bp (insertion C), respectively.

TLR9-1237T>C (NCBI SNP CLUSTER ID: rs5743836) genotyping was performed with TaqMan method. Primers used were: forward primer 5'-GGC CTT GGG ATG TGC TGT T-3' and reverse primer 5'-GGT GAC ATG GGA GCA GAG ACA-3'. Dual-labeled fluorogenic hybridization MGB-probes used were: CTGCCTGAAACT 5' Fluor Label (FAM, 6-carboxyfluorescein) and CTGGAAACTCCCC 5' Fluor Label (VIC).

TLR9+2848G>A genotyping (NCBI SNP CLUSTER ID: rs352140) was performed with TaqMan method. Primers used were: forward primer 5'-CCG GTC TGC AGG TGC TAG AC-3' and reverse primer 5'-CCA AAG GGC TGG CTG TTG TA-3'. Dual-labeled fluorogenic hybridization MGB probes used were: AGCTACCGCGACTGG 5' Fluor Label (FAM) and AGCTACCACGACTGGA 5' Fluor Label (VIC).

Genotyping of the *IRAKM*+22148G>A exon 5 SNP (NCBI SNP CLUSTER ID: rs1152888) was performed by PCR with forward primer 5'-AGT GGA AC T GAT GTC CTG TGA CAG-3' and reverse primer 5'-GCA ACA CAT TGA CCT AAT GAC CAG-3'.

The PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 50 s, at 60 °C for 50 s, at 72 °C for 150 s and a final extension at 72 °C for 5 min followed by cooling to 4 °C. Digestion overnight with *RsaI* (Invitrogen Life Technologies) of the 505-bp amplicons resulted in two fragments of 188+317 bp (allele G) or 505 bp (allele A).

Statistical analysis

Hardy-Weinberg equilibrium was determined in healthy controls to assess the Mendelian inheritance. Comparisons of the genotypes between control and different groups of IPAA patients were performed by Fisher's exact or χ^2 two-tailed tests where appropriate. Carrier trait analysis was performed to determine whether combinations of SNPs were acting synergistically on the risk of developing pouchitis or of predisposing to chronic relapsing pouchitis. Adjusted odd's ratio (OR) and 95% confidence intervals (95%CI) were calculated. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of patients and control groups

The demographic features of IPAA patients and healthy

Table 2 Clinical data obtained from IPAA patients

IPAA patients	Mean (SD)	Median	Range
Age (yr) at diagnosis of ulcerative colitis	29.7 (11.7)	27	8-61
Time (yr) from diagnosis of ulcerative colitis to IPAA surgery	6.3 (5.5)	5	0-34
Time from IPAA surgery to first episode of pouchitis			
Total pouchitis group ($n = 78$)	2.8 (3.1)	2	0-12
Infrequent pouchitis (≤ 2 episodes, $n = 43$)	3.7 (3.3)	3	0-12
Chronic relapsing pouchitis (≥ 3 episodes, $n = 35$)	2 (2.6)	1	0-12
Pattern of pouchitis			
Duration of pouch (yr)			
No episodes of pouchitis ($n = 79$)	6.3 (4.1)	5	0-14
Infrequent pouchitis (≤ 2 episodes, $n = 43$)	7.6 (3.8)	7	1-16
Chronic relapsing pouchitis (≥ 3 episodes, $n = 35$)	7.9 (3.6)	7	2-14

Table 3 Genotypes of the *CD14*, *CARD15*, *TLR4*, *TLR9*, and *IRAKM* gene polymorphisms in subgroups of patients with IPAA and controls

Polymorphisms	Genotype	Controls $n = 224$ (%)	No pouchitis $n = 79$ (%)	Infrequent pouchitis $n = 43$ (%)	Chronic relapsing pouchitis $n = 35$ (%)
<i>CD14</i> -260 C>T	CC	55 (24.6)	24 (30.4)	11 (25.6)	7 (20)
	CT	102 (45.5)	34 (43)	21 (48.8)	22 (62.9)
	TT	67 (29.9)	21 (26.6)	11 (25.6)	6 (17.1)
<i>CARD15</i> 3020InsC	WT/WT	218 (97.3)	77 (97.5)	43 (100)	34 (97.1)
	WT/InsC	6 (2.7)	2 (2.5)	0 (0)	1 (2.9)
	InsC/InsC	0 (0)	0 (0)	0 (0)	0 (0)
<i>TLR4</i> +896 A>G	AA	208 (92.9)	73 (92.4)	38 (88.4)	33 (94.3)
	AG	16 (7.1)	5 (6.3)	5 (11.6)	2 (5.7)
	GG	0 (0)	1 (1.3)	0 (0)	0 (0)
<i>TLR9</i> -1237 T>C	TT	158 (70.5)	52 (65.8)	34 (79.1)	19 (54.3)
	TC	60 (26.8)	24 (30.4)	9 (20.9)	14 (40)
	CC	6 (2.7)	3 (3.8)	0 (0)	2 (5.7)
<i>TLR9</i> +2848 G>A	GG	40 (17.9)	20 (25.3)	9 (20.9)	6 (17.1)
	GA	104 (46.4)	38 (48.1)	22 (51.2)	16 (45.7)
	AA	80 (35.7)	21 (26.6)	12 (27.9)	13 (37.2)
<i>IRAKM</i> +22148G>A	GG	181 (80.8)	64 (81)	33 (76.7)	28 (80)
	GA	42 (18.8)	14 (17.7)	7 (16.3)	7 (20)
	AA	1 (0.4)	1 (1.3)	3 (7)	0 (0)

controls are shown in Table 1. No statistical differences were found in the variables including gender and age between the IPAA group and healthy controls.

The clinical characteristics of patients with IPAA for ulcerative colitis were summarized. Information on the pattern of pouchitis within the group of IPAA patients for ulcerative colitis (patients who did not develop a pouchitis, patients who had infrequent pouchitis and patients who suffered from chronic relapsing pouchitis, respectively) is shown in Table 2. No statistically significant differences were found in gender and age between controls and IPAA patients as well as in the duration of pouch and the first episode of pouchitis after IPAA, respectively and among IPAA groups and between infrequent pouchitis and chronic relapsing pouchitis groups.

Genotyping

The genotype frequencies in the control group were in Hardy-Weinberg equilibrium for the *CD14*, *TLR4*, *TLR9*, *CARD15/NOD2*, and *IRAKM* gene polymorphisms. The genotype frequencies of these polymorphisms are described in Table 3. No significant differences in allele-, genotype- or carrier frequencies of the gene polymorphisms were found between the healthy controls

and IPAA patients.

No significant differences in allele-, genotype- or carrier frequencies of the gene polymorphisms were found between the three subgroups of IPAA patients except that the carriership of allele *TLR9* -1237 C was more frequent in patients with chronic relapsing pouchitis (45.7%) as compared to those with infrequent pouchitis (20.9%) ($P = 0.028$, OR = 3.2, 95%CI = 1.2-8.6). When the combined groups of infrequent pouchitis and chronic relapsing pouchitis (i.e. total pouchitis group) were compared to the patients without pouchitis, carriership of this allele was not significantly different ($P = 0.87$, OR = 1.1, 95%CI = 0.6-2.1).

TLR9 haplotype

The two analyzed *TLR9* SNPs were chosen based on the study of Lazarus *et al.*^[33] in which a set of four frequent *TLR9* SNPs designated as *TLR9* -1486, *TLR9* -1237, *TLR9* +1174, and *TLR9* +2848, were described. Genotyping of both *TLR9* -1237 and *TLR9* +2848 could distinguish all four-locus haplotypes commonly present in the European American population. We therefore calculated the haplotypic genotypes in Italian Caucasians (Table 4). The haplotype frequencies in the healthy controls were identical to the European-Americans as reported by Lazarus *et al.*^[33].

Table 4 Frequencies of *TLR9* haplotypes formed by -1237 T>C and +2848 G>A SNPs

<i>TLR9</i> -1237	<i>TLR9</i> +2848	Haplotype	Controls 2n = 448	No pouchitis 2n = 158	Infrequent pouchitis 2n = 86	Chronic relapsing pouchitis 2n = 70
T	G	I	181 (40)	51 (32)	40 (47)	28 (40)
T	A	II	195 (44)	77 (49)	37 (43)	24 (34)
C	A	III	69 (15)	29 (18)	9 (10) ¹	18 (26) ¹
C	G	IV	3 (1)	1 (1)	0	0

¹P = 0.018, OR = 3.0, 95%CI = 1.2-7.1.

Haplotype III was more frequent in chronic relapsing pouchitis as compared to infrequent pouchitis ($P = 0.018$; OR = 3.0, 95%CI = 1.2-7.1). This haplotype, however, did not show nucleotides uniquely present (tag SNPs) on position -1486 (allele T present in haplotypes I and III) or on position +1174 (allele G present in haplotypes II and III), indicating that allele *TLR9*-1237C could provide the strongest association.

Carrier trait analysis

To investigate if SNPs in different genes could act synergistically on disease susceptibility and/or severity, carrier trait analysis with the associated *TLR9* allele was performed. Simultaneous carriership of alleles *TLR9* -1237C and *CD14* -260T was more frequent in patients with chronic relapsing pouchitis as compared to those with infrequent pouchitis ($P = 0.018$, OR = 4.1, 95%CI = 1.4-12.3), which was more significant as compared to the analysis of allele *TLR9* -1237C alone ($P = 0.028$, OR = 3.2, 95%CI = 1.2-8.6). No other significant carrier traits were observed.

DISCUSSION

There is convincing evidence that enteric bacteria play a role in driving the inflammatory response in IBD and that genetic factors contribute not only to the pathogenesis but also to the course and extent of these disorders. Given these processes, we investigated whether polymorphisms in the following genes encoding for proteins involved in innate immunity, *TLR4* +896 A>G, *TLR9* +2848 G>A, *TLR9* -1237T>C, *CD14* -260C>T, *CARD15/NOD2* 3020insC, and *IRAKM* +22148 G>A, were associated with the development of pouchitis, disease frequency or severity.

Analysis of the three subgroups of IPAA patients (i.e. patients who never developed pouchitis, patients with infrequent pouchitis and patients with a chronic refractory form of pouchitis) revealed a positive association of allele *TLR9* -1237C with the risk of developing chronic refractory pouchitis, once these patients developed pouchitis. Haplotype analysis showed that out of the four SNPs defining *TLR9* haplotypes, this allele was uniquely responsible for this finding. Subsequently, we investigated whether interactions of allele *TLR9* -1237C with SNPs in the other candidate genes might strengthen this association. Carrier trait analysis revealed that an even stronger association was apparent with the combination of alleles *TLR9* -1237C and *CD14* -260T. These data suggest

that this combination of alleles might be a valuable genetic marker to identify a clinical subgroup of IPAA patients with an enhanced risk of developing chronic pouchitis, compared to alleles of the SNPs *TLR4* +896 A>G, *TLR9* +2848 G>A, *CARD15/NOD2* 3020insC, and *IRAKM* +22148 G>A.

It cannot be excluded that the group of patients who did not develop pouchitis consisted of a mixture of patients who never developed pouchitis on the one hand and patients who proceeded to the infrequent or chronic relapsing pouchitis group on the other hand. This could explain why we did not detect an association of allele *TLR9* -1237C between the no-pouchitis and the chronic relapsing pouchitis groups. It should be noted that no significant differences were found in the mean duration of IPAA between the three groups.

At present it is unknown as to what effect of the *TLR9*-1237 T>C SNP exerts on the expression of *TLR9* given its location in the far promoter region where no DNA-binding site for known transcription factors is apparent. The association observed might therefore result from linkage disequilibrium with another polymorphism(s) in a nearby gene.

The mechanism underlying increased risk of developing chronic relapsing pouchitis by a combined carriership of alleles *TLR9* -1237C and *CD14* -260T might be a dysfunction in bacterial recognition or a lack of an adequate immune response to bacterial challenge. This could start at the level of the plasmacytoid dendritic cells, which play a central role in bacterial recognition, selectively express *TLR9* and have soluble *CD14* facilitating reactivity to a broad array of bacterial components^[43] or at the level of the intestinal epithelium. Soluble *CD14* might confer epithelial cell responsiveness^[25].

The regulatory role of dendritic cells is of particular importance in the intestine where the mucosal immune system is closely associated with the external environment^[44]. Dendritic cells sample bacterial products either indirectly via M cells or directly by reaching between epithelial cells into the gut lumen^[45]. In this perspective, it is noteworthy to mention a recent article that reported a lack of immature blood dendritic cells, which possibly migrate to the gut in IBD patients with active disease^[46].

Recently, carriership of the *TLR9* -1237C allele has been associated with Crohn's disease^[34]. Ileal involvement is present in about 60% of patients with Crohn's disease. Since the pouch is an ileal reservoir that is more vulnerable to the continuous contact with high bacterial titers, it could

be hypothesized that carriership of the allele *TLR9* -1237C (with or without *CD14* -260T) is associated with an impaired immune response at the level of the ileal tissue. Paneth cells are located in the crypts and are central in host defense to luminal bacteria by releasing antimicrobial substances^[47,48].

If this is true, carriership of allele *TLR9* -1237C may become an important predictive marker to the enhanced risk of developing refractory chronic pouchitis and eventually pouch failure.

SNPs in different genes might work synergistically and constitute a small to moderate relative risk of developing diseases. Though the observations we described in this article were based on a relatively less number of patients, it should be realized that this study might represent one of the largest series available.

In conclusion, our data suggest that the alleles *TLR9*-1237C and *CD14*-260T synergistically enhance the risk of developing chronic relapsing pouchitis and eventually pouch failure in ulcerative colitis patients who need surgical intervention. Larger studies are required to determine whether this allelic combination becomes a valuable predictive marker and functional studies on the biological role of *TLR9* and *CD14* in pouchitis.

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• RAPID COMMUNICATION •

Impairment of IFN- α production capacity in patients with hepatitis C virus and the risk of the development of hepatocellular carcinoma

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INTRODUCTION

The natural history of chronic hepatitis C virus (HCV) infection entails a gradual progression from chronic hepatitis (CH) to liver cirrhosis (LC) over 10-20 years leading to hepatocellular carcinoma (HCC) within 30 years^[1-3]. Some common factors influencing the development of HCC include the degree of liver fibrosis, old age, sex (male), high alanine aminotransferase (ALT) level and a history of diabetes and alcohol consumption^[4,5]. In addition, the development of HCC is affected by viral factors^[6] and host immune factors^[7]. Although recent progress in hepatitis research has illustrated the role of viral factors, analysis of the role if any played by immune factors has been insufficient. Several investigators have discussed the impairment of NK cell function, cytotoxic T lymphocyte (CTL) function and dendritic cell (DC) function in hepatitis C patients^[8-11].

In previous papers, we have reported about the impairment of HVJ induced IFN- α production in whole blood cultures of patients with lung cancer, myelodysplastic syndrome, pulmonary tuberculosis, HIV, diabetes mellitus and IgA nephritis^[12,13]. Additionally, we mentioned that IFN- α production in patients with lung cancer gradually decreases with the progress of cancer^[12]. In the present study, we examined the IFN- α production in a large number of patients with HCV infection and LC who were monitored for more than 3 years for the development of HCC. IFN- α production in patients who developed HCC within 3 years was significantly lower than that of patients who remained in LC without developing HCC. These results suggest that IFN- α production could be a parameter with promising anti-tumor immuno-surveillance potential, i.e., measurement of IFN- α production in patients with HCV infection may be useful for the early detection of HCC.

Abstract

AIM: To determine the utility of interferon (IFN) - α production capacity in patients with hepatitis C virus (HCV) infection for the measurement of immuno-surveillance potential and for the early detection of hepatocellular carcinoma (HCC) by investigating the Sendai virus (HVJ) stimulated IFN- α production capacity of patients with HCV infection.

METHODS: HVJ stimulated IFN- α production was determined in a large number of patients with HCV infection and the development of HCC was monitored for 3 years in patients with liver cirrhosis (LC).

RESULTS: IFN- α production capacity decreases gradually with the progression of liver disease from chronic hepatitis (CH) to HCC. A significant correlation between the duration of HCV infection and impaired IFN- α production capacity was observed. IFN- α production in patients who developed HCC within 3 years was significantly lower than that of patients who remained in LC without developing HCC.

CONCLUSION: Measurement of IFN- α production in LC patients may be useful for the early detection of HCC.

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Key words: IFN- α production capacity; Hepatitis C virus; Hepatocellular carcinoma

MATERIALS AND METHODS

Subjects

IFN- α production in whole blood cultures was measured

in 155 healthy individuals and 82 patients with chronic HCV infection including CH, LC and HCC. Blood was drawn after receiving informed consent.

The patients with HCV infection were all outpatients at Kyoto University Hospital, and were followed up at least once a month from 1994. All patients tested positive for anti-HCV (Abbot Japan, Tokyo) and/or HCV RNA by RT-PCR. The diagnoses of CH, LC and HCC were made by conducting a comprehensive analysis of liver histology, image analysis and blood tests. Patients underwent abdominal ultrasonography every 3–6 mo and/or contrast enhanced computed tomography every 6–12 mo. The CH group included 27 men and 12 women (age, 48.9 ± 12.2 years). The LC group included 11 men and 10 women (age, 64.0 ± 7.5 years). The HCC group included 13 men and 9 women (age, 64.1 ± 5.4 years). Patients with HCV infection had not received interferon therapy for at least 3 mo prior to the measurement of IFN production capacity. In the case of patients with HCC, the IFN production capacity values of patients used were those determined at the point of first diagnosis of HCC.

We selected age-matched healthy persons as controls for comparison with the patients with HCV infection. Healthy subjects, 30 men (age, 53.6 ± 8.9 years) and 95 women (age, 53.2 ± 7.6 years), were selected from the people receiving medical examinations at the clinic of the Louis Pasteur Center for Medical Research who were over 40 years of age and had no acute and/or chronic disease and no abnormal values in blood tests.

Measurement of IFN- α production capacity in human whole blood

Two milliliters of heparinized blood was cultured with 500 HA U/mL Sendai virus (HVJ) within 5 h after the withdrawal of blood. The blood-virus mixture was incubated at 37 °C for 20 h. Supernatants were harvested by centrifugation at 3 000 r/min for 10 min. IFN- α activity in the supernatants was determined by bioassay as mentioned in a previous paper^[12].

Statistical analysis

Results were expressed as mean \pm SD. Statistical significance was tested by Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) or the χ^2 test was used, when appropriate.

RESULTS

IFN- α production capacity in patients with chronic HCV infection in the various stages

A previous study has reported that there was a significant difference in IFN- α production between men and women, but that the IFN- α production capacity per 1 000 leukocytes (WBC) did not differ significantly between men and women throughout various groups^[12]. In this study, IFN- α production capacity in various stages is expressed as IFN- α production per 1 000 WBC for comparing

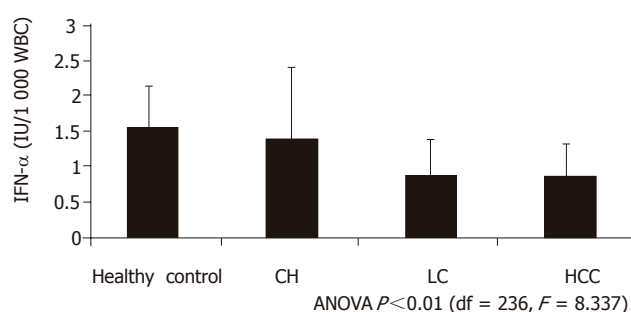


Figure 1 IFN- α production capacity per 1 000 WBC in patients with persistent HCV infection in various stages.

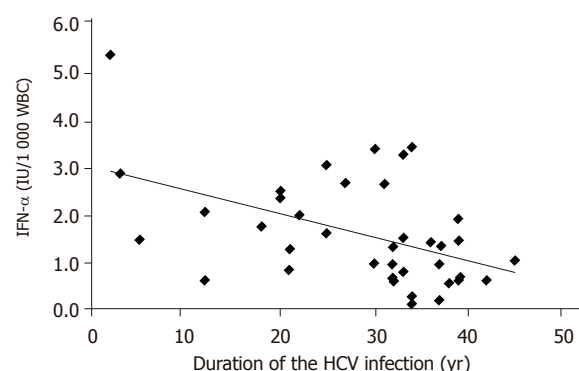


Figure 2 IFN- α production capacity per 1 000 WBC and duration of HCV infection.

each stage, normalized for any difference between men and women. We found that IFN- α production capacity per 1 000 WBC decreased with the progression of the disease (healthy control: 1.54 ± 0.57 ; CH: 1.41 ± 1.01 ; LC: 0.89 ± 0.49 ; HCC: 0.87 ± 0.45 IU/1 000 WBC) (Figure 1). Using ANOVA, we found that HCV groups showed a significantly lower IFN- α titer as compared with the control group.

IFN- α production capacity per 1 000 WBC and the duration from HCV infection by blood transfusion

It has been reported that a significant correlation exists between the incidence of HCC and the duration of HCV infection^[1–3]. As the time of initial infection is precisely known for patients who were infected while undergoing transfusion, we chose HCV patients infected in this manner. We then looked at the relationship between the patient's IFN- α production capacity and the duration of the HCV infection (Figure 2). A significant correlation between the duration of HCV infection and impaired IFN- α production capacity per 1 000 WBC was observed ($n = 39$, $r = 0.48$, $P = 0.0019$). We could not find any significant correlation between platelet number, which was a surrogate marker for liver fibrosis, and ALT and AST levels, which were markers for the activity grade.

Table 1a IFN- α production capacity and the risk of HCC development within 3 years of LC

<i>n</i>		Group A 11	Group B 10
Age	(yr)	63.6 \pm 8.3	64.6 \pm 6.7
IFN- α	(IU/mL)	2 406 \pm 1 239	3 936 \pm 2 284
IFN- α /WBC	(IU/1 000 WBC)	0.66 \pm 0.32	1.14 \pm 0.53 ^a
AST(GOT)	(IU/mL)	97.0 \pm 52.7	55.9 \pm 21.3 ^a
ALT(GPT)	(IU/mL)	99.2 \pm 47.0	54.8 \pm 22.4 ^b
Plt	($\times 103/\mu\text{L}$)	79.8 \pm 44.8	93.6 \pm 39.2

^a $P < 0.05$, ^b $P < 0.01$ **IFN- α production capacity and the risk of HCC development within 3 years of LC**

We measured IFN- α production capacity in the LC group. After 3 years, we divided the original group into two, according to whether each patient developed HCC or not. We observed a significant difference ($P < 0.05$) in the initial IFN- α production capacity per 1 000 WBC between the patients who developed HCC (Group A, $n = 11$) and those who remained in LC without developing HCC (Group B, $n = 10$). In addition, we observed significant differences in AST and ALT levels as well as IFN- α production capacity per 1 000 WBC between the two groups (Table 1a).

Since the mean IFN- α production value of the HCC patients was 0.87 ± 0.45 IU/1 000 WBC, we divided the LC group into two sub-groups: patients with IFN- α production value greater or less than the mean value (0.87 IU/1 000 WBC) (Table 1b). After 3 years, we determined whether each patient had developed HCC or not. Compared to the 22% of patients in the high titer group, 75% of patients in the low titer group developed HCC. The χ^2 test showed significant differences between the two groups ($P < 0.05$). These results indicated that LC patients with a low IFN- α production capacity (< 0.87 IU/1 000 WBC) had a high risk of developing HCC within 3 years. Although low IFN- α production and high AST and ALT values, HCV copies and genotypes were demonstrated as risk factors of HCC development, no correlations were found between IFN- α production and these factors.

DISCUSSION

In this study, we have demonstrated that (1) HVJ-stimulated IFN- α production capacity decreased gradually with the progression of HCV infection from CH to LC to HCC; (2) the longer the duration of HCV infection was, the lower was the patient's IFN- α production capacity; and (3) IFN- α production capacity was lower in patients who developed HCC within 3 years as compared to those who did not develop HCC. Based on these results, a patient with lower IFN- α production capacity might be considered to be at a high risk for the development of HCC.

The progress of chronic HCV infection is one of slow progression from the early stages of CH without fibrosis to LC and HCC^[1-3]. The disease progresses to its advanced

Table 1b Relationship between IFN- α production capacity and incidence of HCC development of patients within 3 years

IFN- α IU/1 000 WBC	No of patients	No of patients who developed HCC within 3 yr	%
≥ 0.87	9	2	22.2
< 0.87	12	9	75 ^a

^a $P < 0.05$

stages over a period of 10-30 years, unless the viral infection is terminated by antiviral therapy^[14]. In this study, we have demonstrated that the IFN- α production capacity of patients with HCV infection gradually decreased as the disease progressed, resulting in the development of HCC, suggesting that extended dysfunction of the type I IFN system may lead to an increased risk of HCC development.

In our previous studies, we have reported that IFN- α production was somehow decreased in patients with lung cancer, MDS, pulmonary tuberculosis, HIV infection, diabetes mellitus and IgA nephritis^[12,13], suggesting that IFN- α production capacity is impaired by various diseases, including cancer, infections, and metabolic disorder. In addition, periodic measurements of IFN- α production revealed decreased IFN- α production capacities in patients with lung^[12]. Consistent with these results, we demonstrated that LC patients who developed HCC within 3 years possessed a decreased capacity to produce IFN- α , suggesting that a low level of IFN- α production is associated with a higher incidence of HCC development in LC patients. In this study, 75% of LC patients with IFN- α production less than 0.87 IU/1 000 WBC developed HCC within 3 years. In the previous study, we have reported the mean values of IFN- α production in lung cancer and MDS to be 0.88 ± 0.80 and 0.35 ± 0.28 IU/1 000 WBC, respectively. These results suggest that patients with IFN- α production less than 0.87 IU/1 000 WBC need to be cautioned that they are at particularly high risk of cancer development. Consistently, MDS patients with low IFN- α production capacity developed cancer within several years^[12,15,16]. Moreover, DM patients with low IFN- α production had a higher risk of developing cancer^[6]. To clarify the utility of this IFN- α production capacity in the early detection of various types of cancers, further large scale clinical surveys are needed.

Why is impaired IFN- α production a risk factor for developing HCC? In our previous study, we have demonstrated that impairment of IFN- α production capacity in whole blood mainly depends not only on a defect in the number of mononuclear lymphocytes but also on the lower capacity of HVJ-stimulated IFN- α production from mononuclear lymphocytes. Mononuclear lymphocytes included monocytes, B cells, NK cells and plasmacytoid dendritic cells, all of which were producers of IFN- α ^[17,18]. Recently, there has been a new interest in type I IFN as a "bridge system" linking innate and adaptive immunity stemming from the identification of natural IFN producing cells, characterized as plasmacytoid dendritic cells^[19,20]. Since IFN- α/β participate in cancer

immunosurveillance^[21], IFN- α production capacity is considered to reflect a facet of individual cancer immunosurveillance potential. In fact, some studies have clearly demonstrated that metastatic tumor cells grow progressively in mice pre-treated with neutralizing antibodies to murine IFN- α/β , demonstrating the critical role of endogenous IFN- α/β in the inhibition of anti-tumor immune responses^[20,22,23]. Furthermore, type I IFN is involved in the anti-tumor activation of NK cells and macrophages as well as the induction of anti-tumor CTLs^[24-27].

IFN- α production capacity is determined by both the genetic background and by the physical condition of each individual, such as the presence/absence of infection, metabolic disorder and cancer^[28]. It is therefore anticipated that periodic measurements of IFN- α production and the early detection of reduced IFN- α production capacity will contribute to the early detection of HCC as well as the early detection of cancer and infectious disease. Furthermore, intervention to increase and/or maintain IFN- α production capacity may be useful for the prevention of HCC development.

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• RAPID COMMUNICATION •

Risk factors for bleeding after endoscopic mucosal resection

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Abstract

AIM: To clarify the risk factors for bleeding after endoscopic mucosal resection (EMR).

METHODS: A total of 297 consecutive patients who underwent EMR were enrolled. Some of the patients had multiple lesions. Bleeding requiring endoscopic treatment was defined as bleeding after EMR. Odds ratios (OR) with 95% confidence intervals (CI), calculated by logistic regression with multivariate adjustments for covariates, were the measures of association.

RESULTS: Of the 297 patients, 57 (19.2%) patients with bleeding after EMR were confirmed. With multivariate adjustment, the cutting method of EMR, diameter, and endoscopic pattern of the tumor were associated with the risk of bleeding after EMR. The multivariate-adjusted OR for bleeding after EMR using endoscopic aspiration mucosectomy was 3.07 (95%CI, 1.59-5.92) compared with strip biopsy. The multiple-adjusted OR for bleeding after EMR for the highest quartile (16-50 mm) of tumor diameter was 5.63 (95%CI, 1.84-17.23) compared with that for the lowest (4-7 mm). The multiple-adjusted OR for bleeding after EMR for depressed type of tumor was 4.21 (95%CI, 1.75-10.10) compared with elevated type.

CONCLUSION: It is important to take tumor characteristics (tumor size and endoscopic pattern) and cutting method of EMR into consideration in predicting bleeding after EMR.

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Key words: Endoscopic mucosal resection; Bleeding; Tumor characteristics; Cutting method

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INTRODUCTION

Endoscopic mucosal resection (EMR) of early gastric cancers has been widely accepted as a standard procedure because of its low degree of invasiveness and the excellent quality of life in patients^[1-3]. Bleeding is one of the major complications of EMR for gastric lesions. The reported bleeding rate after EMR for early gastric cancers is between 1.4% and 20%^[4-8]. As indications for EMR expands, more complications such as bleeding may occur with it. It is reported that patients who required blood transfusion after EMR due to severe bleeding account for 4-14% of all patients undergoing EMR^[7,13]. Several factors related to bleeding after EMR, such as tumor diameter, have been reported^[9]. Our aim is to clarify the risk factors for bleeding after EMR in 297 patients with gastric lesions.

MATERIALS AND METHODS

Study population

A total of 297 consecutive patients who underwent EMR between April 1991 and December 1997 in the Department of Gastroenterology of Osaka City University Medical School were enrolled. Inclusion criteria were all gastric lesions confined to the lamina propria. The depth of tumor invasion was determined by endoscopic ultrasound. There was no bleeding tendency in any of the studied patient, and no patients used drugs such as anticoagulants.

Methods and measurements

The gastric lesions were removed by strip biopsy or endoscopic aspiration mucosectomy. We defined early gastric cancer using the Japanese Classification of Gastric Carcinoma^[9,10], and we have defined the absolute indication

for endoscopic treatment as follows: elevated type mucosal cancer less than 2 cm or depressed type mucosal cancer without ulceration less than 1 cm in size. We have also carried out endoscopic treatment in cases extending beyond these criteria as a relative indication, when we could not perform surgery due to patient's refusal or due to significant heart, lung or kidney failure. Strip biopsy was performed with a two-channel scope (GIF2T-200; Olympus Optical Co., Tokyo, Japan), as described by Tanabe *et al.*^[11]. Physiologic saline was injected locally to elevate the lesion. Endoscopic aspiration mucosectomy was performed as described by Torii^[10]. A snare was introduced through a Teflon tube and tightened around the outer circumference of a transparent plastic cylinder cap (Olympus Optical Co., Tokyo, Japan). The Teflon tube, used as a snare introducer, was taped along the external axis of the endoscope. The snare was tightened by manipulating the handle, just as in polypectomy, and was fixed by taping it to the endoscope so that the snare loop remained at the tip of the cylinder cap. Physiologic saline was injected via an injection needle at the lesion to elevate the lesion. The snare was pushed over the tumor, while the lesion was aspirated along with the surrounding normal mucosa, and resection was performed by electrocauterization. A transparent cap was attached to the tip of an endoscope (GIFQ-200, GIFQ-230, GIFXQ-200, GIFXQ-230; Olympus Optical Co., Tokyo, Japan). The outer diameter of the transparent cap was 14.8 mm (MH-589) or 12.6 mm (MH-587).

Patients with a gastric lesion that required endoscopic treatment for bleeding after EMR were considered to have bleeding after EMR. We examined the patients with bleeding immediately after EMR, because they were treated with anti-ulcer drugs after EMR. Some of the patients had multiple lesions in our study in which the largest lesion per patient was selected as a representative lesion. Furthermore, when a patient had multiple tumors with the same largest diameter, we excluded the patient from analysis. We analyzed the data according to this rule.

The location of the lesion, tumor diameter, endoscopic pattern, histological findings for the tumor and cutting method were examined for their relationship to bleeding after EMR. After EMR, the removed mucosa was semifixed in formalin, and the lesion diameter was measured with a stereoscopic microscope (SZH-ILLB, Olympus Optical Co. Ltd., Tokyo, Japan). Based on the location, gastric lesions of the stomach was classified into an antrum group including the antrum and angle of the stomach, and a corpus group including the lower, middle, and upper body of the stomach. There were 158 lesions in the antrum group and 139 in the corpus group. By endoscopic pattern, lesions were classified into two types: elevated type and depressed type. There were 253 lesions of elevated type and 44 of depressed type. Histological examination revealed 139 early gastric cancers and 158 gastric adenomas^[8]. We removed 177 lesions by the strip biopsy method and 144 lesions by endoscopic aspiration mucosectomy. Informed consent for EMR was obtained from all the patients.

Table 1 Baseline characteristics of study patients

	No bleeding after EMR (<i>n</i> = 240)	Bleeding after EMR (<i>n</i> = 57)	<i>P</i>
Age (yr)	65.3±9.3	66.0±8.4	0.582
Sex (M:F)	170:70	41:16:00	0.998
Tumor factors			
Location			
Antrum group	135 (85.4%)	23 (41.6%)	0.044
Corpus group	105 (75.5%)	34 (24.5%)	
Tumor diameter (mm)	12.4±6.7	13.9±7.1	0.152
Endoscopic pattern of lesion			
Elevated type	211 (83.4%)	42 (16.6%)	0.012
Depressed type	29 (65.9%)	15 (34.1%)	
Histological type			
Borderline adenoma	115 (82.7%)	24 (17.3%)	0.52
Cancer	125 (79.1%)	33 (20.9%)	
Cutting method			
Strip biopsy	156 (88.1%)	21 (11.9%)	0.001
Endoscopic aspiration mucosectomy	84 (70.0%)	36 (30.0%)	

Values are mean±SE or no. of gastric lesions.

Statistical analysis

Values were expressed as mean±SD. Categorical variables were compared using the χ^2 test. Differences in mean values between the two groups were compared using the unpaired *t* test. *P*<0.05 were considered significant. Multiple logistic regression analysis was used to evaluate the simultaneous effects of age, location of lesion, diameter, endoscopic pattern, histology of the tumor and the cutting method of EMR. Linear trends in risk associated with the tumor diameter were evaluated by indicators for each categorical level of tumor diameter using the median value for each category. The 95%CI for each odds ratio (OR) was calculated, and all *P* values are two-tailed. Statistical analyses were made using the SPSS 10.0 software package.

RESULTS

Of the 297 patients, 57 patients (19.2%) were confirmed to have bleeding after EMR. Baseline characteristics of the patients are summarized in Table 1. There was no significant difference in age and gender of patients, tumor diameter or histological type of tumor between the groups. The bleeding incidence after EMR in the corpus group was significantly higher than that in the antrum group (*P* = 0.038). The bleeding rate for depressed type lesions was significantly higher than that for elevated type lesions (*P* = 0.012). The bleeding rate with endoscopic aspiration mucosectomy was much higher than that with strip biopsy (*P*<0.001).

Of the 57 patients with bleeding, 18 (31.6%) patients had spurting bleeding (12 patients underwent EMR by endoscopic aspiration mucosectomy, and 6 underwent EMR by strip biopsy). Of these 57 patients, 39 (68.4%) had oozing bleeding (28 patients underwent EMR by endoscopic aspiration mucosectomy, and 11 underwent EMR by strip biopsy). All bleeding, except in one case, was controlled by endoscopic treatment with endoscopic clipping (HX5LR-1, Olympus, Japan), ethanol injection,

Table 2 Odds ratio of bleeding after endoscopic mucosal resection

	<i>n</i>	Cases	%	Crude-OR (95%CI)	Multiple-adjusted OR ¹ (95% CI)	Multiple-adjusted OR ² (95% CI)
Age (each additional year of age)	297	57	19.2	1.01 (0.98-1.05)	1.00 (0.97-1.04)	1.01 (0.97-1.04)
Tumor factors						
Location of gastric lesions						
Antrum group	158	23	14.6	1	1	1
Corpus group	139	34	24.5	2.24 (1.20-4.18)	2.24 (1.20-4.18)	1.69 (0.88-3.26)
Tumor diameter						
Quartile 1 (4-7)	58	5	8.6	1	1	1
Quartile 2 (8-11)	89	18	20.2	3.15 (1.06-9.38)	3.15 (1.06-9.38)	3.01 (0.99-9.07)
Quartile 3 (12-15)	76	13	17.1	2.53 (0.81-7.87)	2.53 (0.81-7.87)	2.90 (0.91-9.20)
Quartile 4 (16-50)	74	21	28.4	5.35 (1.78-16.10)	5.35 (1.78-16.10)	5.63 (1.84-17.23)
<i>P</i> for trend				0.048	0.022	0.022
Endoscopic pattern of lesion						
Elevated type	253	42	16.6	1	1	1
Depressed type	44	15	34.1	2.60 (1.28-5.26)	3.95 (1.69-9.24)	4.21 (1.75-10.10)
Histological type						
Adenoma	139	24	17.3	1	1	1
Cancer	158	33	20.9	1.27 (0.71-2.27)	0.67 (0.33-1.35)	0.56 (0.27-1.16)
Cutting method						
Strip biopsy	177	17	9.6	1		1
Endoscopic aspiration mucosectomy	120	40	33.3	3.18 (1.75-5.80)		3.07(1.59-5.92)

¹Adjusted for age, tumor factors, location of gastric lesions, tumor diameter, endoscopic pattern of lesion, histological type. AT indicates antrum of stomach;

²Adjusted for age, tumor factors, location of gastric lesions, tumor diameter, endoscopic pattern of lesion, histological type, and cutting method.

and injection of hypertonic saline-epinephrine solution. No patient required blood transfusion in our study, only one patient underwent open emergency surgery because of severe bleeding after EMR.

Multivariate analysis of bleeding risk after EMR

The multivariate analysis of bleeding after EMR is summarized in Table 2. Cutting method of EMR was the strongest factor identified in this analysis. Location of gastric lesion, tumor diameter, and endoscopic pattern of the lesion were associated with an increased risk of bleeding after EMR, when the multivariate analysis was adjusted for age, location of gastric lesions, tumor diameter, endoscopic pattern of lesion and histological type. However, when cutting method of EMR was added to these factors in the multivariate analysis, location of the gastric lesion was no longer a significant factor. The multiple-adjusted OR excluding the factor of the cutting method for bleeding after EMR in the corpus group was 2.24 (95%CI, 1.20-4.18) compared with that in the antrum group. However, the multiple-adjusted OR including the factor of cutting method for bleeding after EMR in the corpus group was 1.69 (95%CI, 0.88-3.26) compared with that in the antrum group.

Tumor diameter and endoscopic pattern of the lesion were not significant factors in the multiple-adjusted analysis, either including or excluding the factor of cutting method. To examine in detail the risk of tumor diameter, tumor diameter was divided into four categories. The multiple-adjusted OR including the factor of cutting method for the highest quartile of diameter was 5.63 (95%CI, 1.84-17.23) compared with that for the lowest.

Concerning endoscopic pattern of lesions, the multiple-adjusted OR for depressed type lesions with the inclusion of the factor of cutting method was 4.21 (95%CI,

1.75-10.10) compared with that for elevated type lesions. The multiple-adjusted OR for bleeding after EMR with endoscopic aspiration mucosectomy was 3.07 (95%CI, 1.59-5.92) compared with that for strip biopsy.

On crude analysis, the histology of the lesion was also associated with the risk of bleeding after EMR, but in the multiple-adjusted analyses this association was not significant.

DISCUSSION

EMR is an established treatment for early-stage gastric cancers, and is an alternative to surgery for patients with superficial neoplastic lesions of the digestive tract. EMR can achieve a complete resection in a majority of patients, but is associated with a higher risk of bleeding than standard polypectomy^[4-7,13,14]. Bleeding incidence, however, appears to vary according to how bleeding is defined. Bleeding during EMR of colorectal polyp is common, occurring in 24% of polypectomy of large colorectal polyps^[15]. Morales *et al.*^[15] defined procedural bleeding as a complication. Various forms of endoscopic treatment, such as multipolar electrocoagulation, use of heat probe, and injection therapy, are usually highly effective in stopping acute upper gastrointestinal ulcer bleeding^[16-18]. Similarly, bleeding during the performance of EMR can almost always be controlled by the injection of saline epinephrine solution, thermal coagulation, or endoscopic clipping^[25]. Endoscopic clipping is believed to be the safest therapeutic modality for controlling spurting bleeding after EMR^[19,20,23]. However, it is reported that patients who required blood transfusion after EMR due to large bleeding accounted for 4-14% of patients undergoing EMR^[7,13].

We came across one patient who underwent open emergency surgery because of severe bleeding after EMR.

Most bleeding occurs during the procedure, although delayed bleeding (up to 3 d in 10% of patients) has been reported^[15]. In our study, patients with a gastric lesion that required endoscopic treatment for bleeding after EMR were considered to be cases to have bleeding after EMR.

The association of various factors with bleeding rates, such as tumor diameter^[13], diameter of the removed mucosa, cutting method^[6,12,13,21], location of gastric lesions, endoscopic pattern of lesions^[13], and histological type of gastric tumor has been reported^[6]. Those studies examined not only the risk of bleeding but also the rate of bleeding.

We found that tumor diameter, endoscopic pattern of lesions, and cutting method were associated with the increased risk of bleeding after EMR. These associations existed even on multivariate analysis.

It has been reported that larger diameter tumors bled readily after EMR^[13]. We evaluated linear trends in risks of tumor diameter by indicators for each categorical level, and found that the findings were consistent with our results from the previous reports. We used tumor diameter determined by postfixation measurement as a predictor of post-EMR bleeding, because formalin fixation did not cause shrinkage of the specimens^[22-23].

No significant relationship was found between bleeding incidence and the cutting diameter^[12], and large cutting diameter was easy to bleed, because bleeding incidence may depend on the size of the cutting diameter^[24]. We did not evaluate the diameter of the removed mucosa because this factor could not be measured before EMR was undertaken, and therefore could not be a predictor of bleeding.

Submucosal injection is an important part of the EMR procedure. Injection of fluid into the submucosa beneath a gastric lesion increases the distance between the base of the polyp and the deeper tissues of the gastric wall. It has been reported that a large submucosal cushion of saline solution increases the safety of polypectomy by preventing thermal injury to these deeper tissues^[26,27]. Therefore, submucosal injection may influence bleeding after EMR. However, effects of the volume of physiologic saline solution injected were not investigated in our study, because the administration volume of physiologic saline solution was not routinely recorded. The endoscopic pattern of the lesion influences the risk of bleeding after EMR has not been reported. Depressed type lesion was a risk factor for bleeding after EMR in our study. However, the volume of submucosal injection used may have affected this result.

There is one report that bleeding incidence is higher with strip biopsy than with endoscopic aspiration mucosectomy, a finding inconsistent with our results^[21]. It was reported that the size of resected specimens was significantly larger with endoscopic aspiration mucosectomy than with strip biopsy^[11]. We did not evaluate the diameter of the removed mucosa in our study, though it might influence the bleeding rate associated with the cutting method.

Blood vessel diameter in the antral region of the stomach is smaller than that in the gastric corpus, and the number of blood vessels in the antral region is

low^[27]. In our study, no significant relationship was found between the locations of the lesion and cutting method in multivariate analysis. However, the bleeding incidence in the corpus group [24.5% (34/139)] was higher than that in the antrum group [14.6% (23/158)]. Therefore, if the sample size of our study was enlarged, a significant difference might be found.

Of the 297 patients, 64 (21.5%) patients underwent EMR by piecemeal resection, with a bleeding incidence of 15.6% (10/64). A total of 233 (78.5%) patients underwent EMR by en-bloc resection, with a bleeding incidence of 20.2% (47/233). There was no significant relationship between *en-bloc* resection or piecemeal resections and bleeding incidence after EMR.

In our study, ten endoscopists performed EMR for patients with bleeding after EMR. Categorical variables, such as location of the lesion, tumor diameter (four categories), endoscopic pattern, histology of the tumor and cutting method were compared among the 10 endoscopists using the χ^2 test. There was no significant difference among the endoscopists in the location of lesion ($P = 0.618$), tumor diameter ($P = 0.182$), endoscopic pattern ($P = 0.374$), histology of the tumor ($P = 0.395$) or cutting method ($P = 0.138$). Therefore, the particular endoscopist performing the procedure did not influence the risk of bleeding.

Conio *et al.* divided the definition of "bleeding after EMR" into three categories, i.e., intraprocedure (occurring during EMR), early (within 24 h), and delayed (≥ 24 h). In our study, "bleeding after EMR" was defined as intraprocedure.

In conclusion, our study indicated that tumor diameter, endoscopic pattern of lesions, and cutting method are risk factors for bleeding after EMR. It is important to consider these three factors in predicting bleeding after EMR.

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• RAPID COMMUNICATION •

Stool antigen tests in the diagnosis of *Helicobacter pylori* infection before and after eradication therapy

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tests performed well. After eradication therapy, negative results were highly accurate for all the three tests. HpStAR had the best overall performance.

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Key words: Diagnosis; Feces; *Helicobacter pylori*; Therapy

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Abstract

AIM: To evaluate two enzyme immunoassay-based stool antigen tests, Premier Platinum HpSA and Amplified IDEIA HpStAR, and one rapid test, ImmunoCard STAT! HpSA, in the primary diagnosis of *Helicobacter pylori* (*H. pylori*) infection and after eradication therapy.

METHODS: Altogether 1 574 adult subjects were screened with a whole-blood *H. pylori* antibody test and positive results were confirmed with locally validated serology and ¹³C-urea breath test. All 185 subjects, confirmed to be *H. pylori* positive, and 97 *H. pylori*-negative individuals, randomly selected from the screened study population and with negative results in serology and UBT, were enrolled. After eradication therapy the results of 182 subjects were assessed.

RESULTS: At baseline, the sensitivity of HpSA and HpStAR was 91.9% and 96.2%, respectively, and specificity was 95.9% for both tests. ImmunoCard had sensitivity of 93.0% but specificity of only 88.7%. After eradication therapy, HpSA and HpStAR had sensitivity of 81.3% and 100%, and specificity of 97.0% and 97.6%, respectively. ImmunoCard had sensitivity of 93.8% and specificity of 97.0%. HpSA, HpStAR, and ImmunoCard had PPV 77%, 80%, and 75%, and NPV 98%, 100%, and 99%, respectively.

CONCLUSION: In primary diagnosis, the EIA-based

INTRODUCTION

H. pylori infection is one of the most common infections in human beings worldwide, strongly associated with peptic ulcer disease and gastric cancer^[1,2]. There are several methods available to detect *H. pylori* infection including invasive methods based on gastric biopsies and non-invasive methods like serology, urea breath tests (UBTs), and stool antigen tests^[3,4]. Stool antigen tests have recently been welcomed with great expectations as they are convenient to the patients and can be easily performed even in small laboratories^[5,6]. However, the accuracy of stool antigen tests in different clinical situations and outside of controlled studies is a matter of concern^[7-9].

Several commercial stool antigen tests are available: enzyme immunoassays (EIAs) based on either polyclonal (Premier Platinum HpSA, Meridian Inc., Cincinnati, OH, USA) or mAbs (Amplified IDEIA HpStAR, also known as Femtolab, Dako, Glostrup, Denmark) and rapid bed-side tests like ImmunoCard STAT! HpSA (Meridian Bioscience Europe, Milan, Italy). HpSA is the most widely studied of these tests and it has shown acceptable performance in the primary diagnosis of *H. pylori* infection but the accuracy in post-treatment setting and in special clinical situations (e.g. upper gastrointestinal hemorrhage, gastric surgery, PPI therapy) has been controversial^[5,7,8]. The three stool antigen tests have rarely been compared in parallel in the same study. The differences in patient characteristics (e.g. peptic ulcer *vs* gastritis) and widely variable prevalence of *H. pylori* in different studies make the comparisons between the tests difficult as the statistical parameters of the performance of the tests are often dependent on the prevalence of the infection.

Recently, we evaluated the three stool antigen tests in *H pylori*-positive patients before and after eradication therapy^[10]. In the present study we also included *H pylori*-negative individuals. *H pylori* infection in all the subjects was verified by serology and ¹³C-UBT.

MATERIALS AND METHODS

Subjects

Adults were invited with a newspaper announcement to participate in the study. Exclusion criteria were antibiotic treatment during previous 2 mo; use of H₂-receptor antagonists, bismuth or proton pump inhibitor therapy during previous 2 wk; *H pylori* eradication therapy during previous 5 years; gastric surgery; chronic gastrointestinal diseases; contraindications to drugs used in the study; pregnancy or lactation. Study subjects were screened for *H pylori* infection with rapid whole-blood antibody tests (Pyloriset Screen II, Orion Diagnostica, Espoo, Finland, or Biocard *Helicobacter pylori* IgG, AniBiotech Ltd, Vantaa, Finland). The present study was subsidiary to another study investigating the efficacy of probiotics in the eradication therapy of *H pylori*. Therefore, allergy to fruit juice containing the probiotics also belonged to the exclusion criteria.

The population screened comprised 1 574 subjects. Positive results in screening were confirmed with UBT and an in-house EIA-based serologic method. Of the 300 *H pylori*-positive subjects in screening, 196 were positive in both of the confirmatory tests, but 11 subjects met one of the exclusion criteria; thus 185 *H pylori*-positive subjects were included in the study (of the remaining 104 subjects positive in screening, 49 were negative in both of the confirmatory tests and for the rest either no confirmatory tests were performed or the results were discordant). From the subjects with *H pylori*-negative test result in screening, 114 (randomly selected by a computer program SPSS 12.0) were invited to bring the specimens for the confirmatory tests and 97 subjects followed this invitation. For these *H pylori*-negative study subjects, the infection was excluded with both of the confirmatory tests. Thus, the final study population consisted of 282 subjects: 185 were *H pylori* positive and 97 negative. The median age of the subjects was 52 years, range 23–71 years. The median age of *H pylori*-positive subjects was 55 years (range 25–71 years) and of *H pylori*-negative subjects 43 years (range 23–64 years). In total there were 186 females and 96 males.

H pylori eradication therapy

A total of 185 *H pylori*-positive subjects received the same eradication therapy: amoxicillin 1 g b.i.d., clarithromycin 500 mg b.i.d. and lansoprazole 30 mg b.i.d. for 1 wk. Exclusion criteria for the therapy were penicillin allergy, prolonged QT-interval, and antifungal therapy for dermatophyte infection.

Serology

At baseline and 4 mo after the end of eradication therapy

the subjects gave serum samples for locally validated in-house EIA^[11]. At baseline, serum samples had been investigated in order to confirm the infection, and they were stored at -20 °C until further used. Serum samples before and after eradication therapy were analyzed in parallel for both immunoglobulin G (IgG) and IgA antibodies as described earlier^[12]. *H pylori* eradication therapy was considered successful when antibody titers of at least IgG class had fallen 40% or more from the pre-treatment level^[11].

UBT

UBT was performed at baseline and 4 wk after the end of eradication therapy. Diabact tablets were used (Diabact UBT, Diabact AB, Uppsala, Sweden)^[13,14]. After overnight fast the subjects swallowed either one or two 50 mg Diabact tablets and blew into the test tube before and 10 min after ingesting the tablet(s). Results were analyzed by isotope ratio mass spectrometry (BreathMATplus, Finnigan MAT GmbH, Bremen, Germany) and expressed as delta over baseline (DOB). Cut-off point for positive test result was DOB 2.2‰.

Stool antigen tests

All subjects gave stool specimens at baseline and those treated 4 wk after the end of eradication therapy. Stool specimens were stored at -20 °C before analysis and were thawed twice at most. Stool samples before and after therapy were always run in parallel in all three antigen tests.

The polyclonal antibody-based Premier Platinum HpSA test, later HpSA (Meridian Inc., Cincinnati, USA) was performed according to manufacturer's instructions. Diluted stool specimens were added to the microtiter wells with a peroxidase-conjugated polyclonal antibody. After washing, substrate was added. The results were read at 450 nm by a spectrophotometry (Titertek Multiskan analyzer, Eflab Oy, Helsinki, Finland). Optical density (OD) values <0.140 were negative, 0.140–0.159 equivocal (gray zone) and ≥0.160 positive, as suggested by the manufacturer. In cases with gray zone values, the same stool samples stored at -20 °C were retested as recommended.

For the mAb-based Amplified IDEIA HpStAR test, later HpStAR (also known by name FemtoLab, Dako), the supernatant of stool suspension and peroxidase-conjugated mAbs were pipetted into the wells. After washing substrate was added, and the results were read by spectrophotometry. According to manufacturer's instructions, OD values ≥0.190 (450 nm) were assessed as positive and <0.190 as negative.

ImmunoCard STAT! HpSA-test, later ImmunoCard (Meridian Bioscience Europe) is based on monoclonal *H pylori* antibodies and a lateral flow chromatography technique. The diluted stool sample was dispensed to the sample port of the test cassette, and after incubation of 5 min at room temperature, the appearance of a pink-red line in the reading window indicated a positive result.

The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the studies and all subjects

Table 1 Performance of the three stool antigen tests as compared with UBT and serology in 282 subjects at baseline

UBT, serology	HpSA	HpStAR	ImmunoCard
Positive 185	Positive 170 ¹	178	172
	Negative 13 ¹	7	13
	2 gray zone ²		
Negative 97	Negative 93	93	86
	Positive 4 ³	4	11

¹One subject originally had gray zone result.²Gray zone result even in re-examination.³Three subjects originally had gray zone results.**Table 2** Performance of the three stool antigen tests as compared with UBT and serology in 182 subjects after eradication therapy

UBT, serology	HpSA	HpStAR	ImmunoCard
Negative 166	Negative 161 ¹	162	161
	Positive 4	4	5
	1 gray zone ²		
Positive 16	Positive 13	16	15
	Negative 3 ³	0	1

¹Six subjects originally had gray zone result.²Gray zone result even in re-examination.³One subject originally had gray zone result.

gave their written informed consent.

RESULTS

The performance of the stool antigen tests at baseline is shown in Table 1 and after eradication therapy in Table 2. Four patients were treated with antimicrobials after eradication therapy before the 4 wk had elapsed and thus, stool specimens and UBTs of these particular patients were not collected until they gave the serum sample for serology 4 mo after the therapy. For all the other subjects, UBT was performed and stool specimens were collected 4 wk after therapy according to the study protocol.

The results after eradication therapy are presented for 182 subjects; 3 subjects had the results only at baseline. One patient died before the follow-up. Serum sample was collected and another subject discontinued the study during antimicrobial therapy because of severe headache, which later turned out to be viral meningitis as examined by a neurologist. After eradication therapy, the confirmatory tests (UBT and serology) showed concordant results in all but one subject, who had eradication failure according to the UBT and all three stool antigen tests but successful eradication according to the serology: his results after eradication therapy were excluded from the analysis.

The performance of the tests at baseline is presented in Table 3 and after eradication therapy in Table 4. The adjusting of the cut-off points would not have had any beneficial effect to the results either before or after eradication treatment (data not shown). The false-positive test results in stool antigen tests were randomly distributed between the individual tests. However, half of the false-negative HpSA test results at baseline were also negative with the other two stool tests.

The HpSA tests with gray zone values were re-examined

Table 3 Performance of the three stool antigen tests at baseline

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HpSA	91.9	95.9	97.7	87.7	93.3
HpStAR	96.2	95.9	97.8	93.0	96.1
ImmunoCard	93.0	88.7	94.0	86.9	91.5

PPV, positive predictive value; NPV, negative predictive value.

Table 4 Performance of the three stool antigen tests after eradication therapy

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HpSA	81.3	97.0	76.5	98.2	95.6
HpStAR	100	97.6	80.0	100	97.8
ImmunoCard	93.8	97.0	75.0	99.4	96.7

PPV, positive predictive value; NPV, negative predictive value.

using the same stool specimens according to manufacturer's recommendations. However, no new stool specimen was collected (although recommended by the manufacturer) if the result was in gray zone even in re-examination. Out of the 464 samples analyzed, 15 (3.2%) fell into the gray zone but only three were equivocal when re-tested. Gray zone results obtained after re-examination were considered neither true positive nor true negative in statistical calculations.

DISCUSSION

In the primary diagnosis of *H. pylori* infection, the EIA-based HpSA and HpStAR tests had high sensitivities in accordance with our previous study^[10] and those of others^[6,8]. Earlier the specificities of these tests have varied between 63% and 100% for HpSA and 87% and 100% for HpStAR in studies with high prevalence of *H. pylori* infection^[6,8,15-19] and are in line with the specificity of 96% in our present study population. ImmunoCard had a good sensitivity, 93.0%, but a low specificity, 88.7%, at baseline, leading to a high number of false-positive test results. In previous studies, ImmunoCard has shown variable results in the primary diagnosis with sensitivities between 83% and 96% and specificities between 82% and 94%^[8,10,20-25].

The positive predictive values (PPVs) for all the three stool antigen tests were very high (94.0-97.8%) at baseline in our study population with a high prevalence (65.6%) of *H. pylori*. However, if these stool antigen tests had been used to detect the infection in the original group of 1 574 screened study subjects with the prevalence of *H. pylori* being 12% (in assuming that all the screened but not confirmed *H. pylori*-negative subjects would have been negative also in the both confirmatory tests), the PPVs for these tests would have been only as follows: HpSA 76.6%, HpStAR 77.5%, and ImmunoCard 54.8%. It has been earlier suggested that at an *H. pylori* prevalence of lower than 30%, the most cost effective diagnostic strategy would be to use stool antigen test and confirm positive results with UBT^[4]. In our study, the ImmunoCard test had the lowest specificity and thus a high number of false-positive test results emphasizing the need for a confirmation of a

positive test result by UBT or serology.

In post-eradication setting, the position of these stool antigen tests is most controversial^[7,8]. This is true especially for the most widely studied HpSA test, which also in our study showed a low sensitivity after eradication therapy. We had an exceptionally low eradication failure rate in this study, only 8.8%, and HpSA test was unable to find 19% of our eradication failures, which is in accordance with our previous endoscopy-based study, in which 25% of eradication failures were unidentified with this test^[10]. For the HpStAR test, the sensitivity has varied after eradication therapy between 86% and 100%, specificity 95% and 100%, PPV 83% and 100%, and NPV 96% and 100%^[8,18,19]. Our results fell well into this range. The performance of ImmunoCard after eradication therapy in the few published studies available has varied widely. In a study in adults ImmunoCard showed very low sensitivity both before (83%) and after (73%) eradication therapy but high specificity 98%^[23] whereas in the study of Gatta *et al.*^[21] the sensitivity was 92% and specificity 100%. In our study all the three stool antigen tests had false-positive results in 2.5%–3.1% of successfully eradicated subjects, figures comparable with UBT^[3,26,27].

All patients except three in our study brought the follow-up UBT and stool specimens 4 wk after finishing the eradication therapy. In some previous studies using the HpSA test, it has been shown that the time elapsed after eradication therapy should not be less than 4 wk but extending the time beyond that would not clearly improve the results^[8,10,19,28,29]. A positive HpSA test as early as 3 d after finishing eradication therapy has been highly predictive of eradication failure but a negative test result was reliable only after 3 wk^[29]. Therefore, it is unlikely that after treatment a longer time before the collection of stool samples would have improved the results in our study.

In conclusion, in the primary diagnosis of *H pylori* infection, the EIA-based HpSA and HpStAR stool antigen tests performed well. However, in a population with a lower prevalence of *H pylori* infection, positive results even in these tests may be reasonable to confirm with UBT or serology. After eradication therapy, negative results were highly accurate for all the three tests and HpStAR even found all the subjects with an unsuccessful eradication. HpStAR had the best overall performance before and after eradication therapy.

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• RAPID COMMUNICATION •

Disrupted NF- κ B activation after partial hepatectomy does not impair hepatocyte proliferation in rats

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Abstract

AIM: To analyze the effects of NF- κ B inhibition by antioxidant pyrrolidine dithiocarbamate (PDTC) or TNF inhibitor pentoxifylline (PTX) on liver regeneration after partial hepatectomy (PH).

METHODS: Saline, PDTC or PTX were injected 1 h before PH and rats were killed at 0.5 and 24 h after PH. Several control groups were used for comparison (injection control groups).

RESULTS: Compared to saline injected controls, NF- κ B activation was absent 0.5 h after PH in rats treated with PDTC or PTX. At 24 h after PH, DNA synthesis and PCNA expression were identical in treated and control rats and thus occurred irrespectively of the status of NF- κ B activation at 0.5 h. Signal transducer and activator of transcription 3 (Stat3) activation was observed already 0.5 h after PH in saline, PDTC or PTX group and was similar to Stat3 activation in response to injection without PH.

CONCLUSION: These data strongly suggest that (1) NF- κ B p65/p50 DNA binding produced in response to PH is not a signal necessary to initiate the liver regeneration, (2) Stat3 activation is a stress response unrelated to the activation of NF- κ B. In conclusion, NF- κ B activation is not critically required for the process of liver regeneration after PH.

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Key words: Partial hepatectomy; Nuclear factor kappa B; Signal transducer and activator of transcription 3; Hepatocyte proliferation; Antioxidant

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INTRODUCTION

The mechanisms initiating the regenerative response after partial hepatectomy (PH) remain controversial. Several lines of evidence point to the critical role of the activation of nuclear factor kappa B (NF- κ B)^[1,2]. This is one of the earliest events constantly detectable in the liver after PH and the previous findings have advocated NF- κ B being a critical effector of the cascade initiating the regenerative process^[3,4]. Some authors also emphasize an anti-apoptotic role of NF- κ B during the regenerative process^[5]. Mice lacking TNF receptor type 1 (TNF-R1) showed deficient NF- κ B binding, low interleukin-6 (IL-6) production, decreased signal transducer and activator of transcription 3 (Stat3) activation and low levels of hepatocyte DNA replication after PH^[6-8]. Based on this and similar observations, it has been proposed that activation of the NF- κ B pathway is a critical step to usher cells into the cell cycle in response to PH. This process might also require the sequential activation of TNF/NF- κ B/IL-6 and Stat3. However, recent studies using mice lacking the common signal transducer of all IL-6 family members gp130 showed only minor effects on the cell cycle and on the peak of DNA synthesis after PH despite an abolished acute phase response and inhibition of Stat3^[9]. Moreover, recent studies in a model of transgenic mice, with specific inhibition of NF- κ B at the hepatocyte level, did not impair DNA synthesis and did not increase liver apoptosis^[10]. In order to clarify the importance of the activation of NF- κ B in the liver regeneration process, we have evaluated the consequences of NF- κ B inhibition on downstream activation of Stat3 and DNA synthesis after PH. Numerous exogenous and endogenous stimuli are capable of inducing NF- κ B activity^[11], but the pathways leading to NF- κ B activation are complex. One of them involves oxidative stress with elevated level of reactive oxygen species^[12,13]. We have therefore administered the antioxidant pyrrolidine dithiocarbamate (PDTC), a compound with metal chelator properties that has been used as a reversible inhibitor of NF- κ B *in vitro* and *in vivo*^[14-17]. Increased production of TNF- α is a potential activator of NF- κ B after PH^[4], although the source of TNF- α after

PH has not been entirely elucidated. To reduce TNF- α production, we administered pentoxifylline (PTX), a methylxanthine derivative, which has been demonstrated to suppress LPS-induced TNF- α production^[18-20]. It may also modulate the expression of other cytokines like IL-6 and IL-1^[21,22].

MATERIALS AND METHODS

Animals

Male Wistar rats (220-270 g body weight) were obtained from the Rat Breeding Facilities of the Catholic University of Louvain Medical School, Brussels, Belgium. All animals were kept in a temperature- and humidity-controlled environment in a 12 h light-dark cycle. At all times, they were allowed free access to water and standard food pellet diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France). The animals were handled according to the guidelines established by the Catholic University of Louvain.

Surgical procedures and experimental design

All animal manipulations were carried out under light ether anesthesia at room air between 9:00 a.m. and 12:00 noon with the use of a clean, but non-sterile technique. PH consisted in mid-ventral laparotomy and resection of the left lateral and median lobes (70% of the liver), according to Higgins and Anderson^[23]. Saline, PDTC (100 mg/kg) and PTX (100 mg/kg) were injected intraperitoneally (i.p.) to the rats 1 h before PH. The rats were killed under ether anesthesia by exsanguination after the puncture of the abdominal aorta and transection of the inferior vena cava in the thoracic cavity, at 0.5 h after PH, i.e. 1 h 30 min after the initial injection of saline or the active compound, and 24 h after PH. Several additional control groups were used for comparison: (1) rats administered saline, PDTC or PTX without PH and killed 1 h after the injection (injection control group), (2) naïve rats that received neither injection nor PH (true controls). The livers were removed, lobes were rapidly weighed, snap frozen in liquid nitrogen and stored at -80 °C. A minimum of three rats was killed in each group at each of the indicated time points.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as previously described^[24]. Protein content was determined using a bicinchoninic acid (BCA) protein assay with serum albumin as a standard (Pierce Chemical, Rockford, IL, USA).

Six to ten micrograms of nuclear proteins were pre-incubated for 10 min at room temperature with 2 μ g poly (dI-dC) in the following binding buffers: NF- κ B (20 mmol/L Hepes, 60 mmol/L KCl, 5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 0.5 mmol/L DTT, glycerol 10%, Nonidet P40 1%); Stat3 (10 mmol/L Hepes, 50 mmol/L NaCl, 1 mmol/L EDTA, glycerol 10%). Double stranded oligonucleotides were ³²P end-labeled with γ -³²P ATP and added to the extracts (10⁵ cpm).

The mixtures were incubated for 30 min at room temperature and then electrophoresed (200 V, 2 h) on a 5% polyacrylamide gel in a 1 \times TBE buffer (25 mmol/L Tris-HCl, 25 mmol/L boric acid, 0.5 mmol/L EDTA). To confirm the identity of the protein/DNA complex, supershift analysis was performed: 4 μ L of specific antibody (1 μ g/ μ L) was added to the samples after 30 min of incubation with the labeled probe and incubated for a further 30 min. Polyclonal antibodies against NF- κ B components p50 and p65, but also p52, c-rel, and rel-B, and anti-Stat3 were purchased from Santa Cruz (CA, USA). The following probes were used: chromatography-purified double stranded oligonucleotides from the class I major histocompatibility complex enhancer element H2kB; TCGAGGGCTGGGGATCCCC CATCTC (NF- κ B) and from the serum-inducible factor binding element in the c-fos promoter; CCAGCATTTCCCGTAAATCCTCCAG; (Stat3). A rabbit reticulocyte (Promega Benelux, Leiden, Netherlands) and an EGF-stimulated A431 cell nuclear extract (Santa Cruz Biotechnology) were used as standards for NF- κ B and Stat3, respectively. Gels were dried and exposed to a Kodak Biomax MS film (NENTM Life Science Products, Inc., Boston, MA, USA) for 16 to 24 h.

Thymidine incorporation

One hour before being killed, 50 μ Ci of [³H]-Thymidine (Amersham, Buckinghamshire, UK) was administered into the femoral vein under light ether anesthesia. At the time of killing (24 h after PH), livers were removed, weighed and rapidly snap frozen in liquid nitrogen and stored at -80 °C. Hepatic DNA synthesis was evaluated by measuring the incorporation of [³H]-Thymidine into the DNA. Total hepatic DNA was extracted as previously described^[25], and incorporation of radioactive nucleotide was measured in a liquid scintillation counter (Wallac 1409, Turku, Finland). Its value is expressed as disintegrations per minute (dpm) per μ g of DNA. All samples were analyzed in duplicate.

PCNA labeling index

Sections from formalin fixed liver tissue (5 μ m thick) were air-dried at 37 °C overnight and dewaxed. Slides were incubated for 30 min in H₂O₂ 0.3% to inhibit endogenous peroxidases, then in TBS containing 10% normal goat serum (NGS, APP Products Ltd, West Midlands, UK) to block non-specific binding sites. Slides were incubated in a monoclonal primary anti-PCNA mouse antibody (PC10, Dako, Denmark; 1:100; overnight), and after washing, with the secondary antibody (anti-mouse antibody; Boehringer, Mannheim, Germany; 1:500; 30 min) followed by streptavidin peroxidase (Boehringer, Mannheim, Germany; 1:1 000; 30 min). Peroxidase activity was revealed by immersion of the slides for 10 min in a 3,3-diaminobenzidine hypochloride solution (DAB 50 mg/100 mL, pH 7.4, Amersham, Cardiff, UK) supplemented with 0.02% H₂O₂. All slides were treated simultaneously to ensure homogeneity of the technique. PCNA labeling index was obtained by examining 3-5 high-power fields in three rats per group. Each field was divided into four zones,

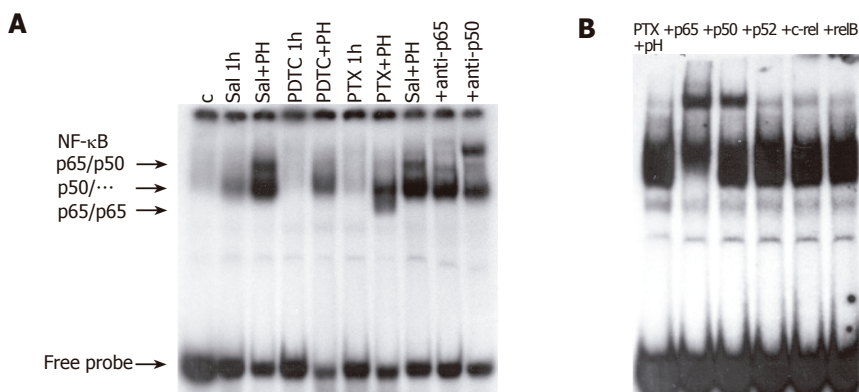


Figure 1 Effect of saline, PDTC, and PTX on NF- κ B binding activity. **A:** Nuclear extracts were prepared from non-treated rats (control), from rats injected with saline, PDTC or PTX subjected or not to partial hepatectomy (PH). No NF- κ B DNA binding activity was observed in the liver nuclear extracts of non-treated rats (controls) nor 1 h after injection of PDTC or PTX. Faint DNA binding was noticed 1 h after the injection of saline which mainly corresponds to NF- κ B p50. Thirty minutes after PH, strong DNA binding was observed in saline-treated groups and two complexes were identified. Supershift experiments identified the upper complex in saline + PH as p65/p50. In the PTX group, a third complex was present 0.5 h after PH, which migrated faster than the dose obtained after saline; **B:** Detailed supershift experiments showed that this faster migrating complex in the PTX+PH group was completely supershifted with the p65 antibody. p52, c-rel and rel-B had no influence on the NF- κ B DNA binding activity.

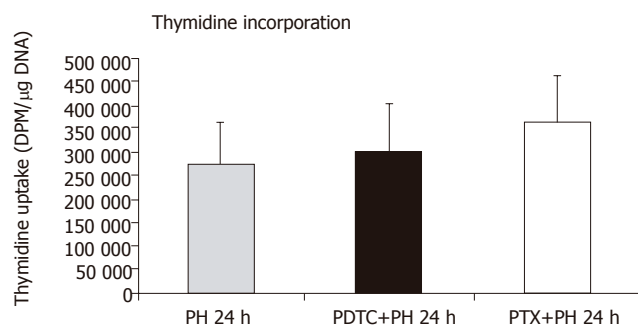


Figure 2 Thymidine incorporation. Similar thymidine incorporation was obtained 24 h after PH in rats pre-treated with an injection of PDTC or PTX compared to non-treated partial hepatectomized rats.

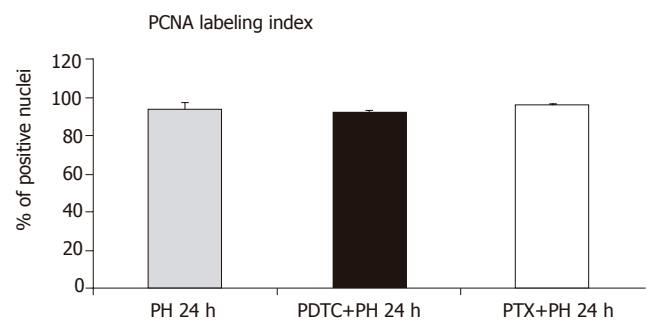


Figure 3 PCNA labeling index. Similar PCNA labeling index was obtained 24 h after PH in rats pre-treated with an injection of PDTC, or PTX compared to non-treated partial hepatectomized rats.

zone 1 being closest to the centrolobular vein and zone 4 corresponding to the periportal areas. Labeling index was defined as the ratio between marked cells and total counted cells.

The same experimented pathologist examined all the slides carefully to detect evidence of necrosis or apoptosis.

Statistical analysis

Results were expressed as mean \pm SE. The statistical differences between the groups were tested using the one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple comparison tests. Statistical significance was admitted for a *P* value of <0.05.

RESULTS

Effect of saline, PDTC, and PTX on NF- κ B binding activity (Figure 1A)

NF- κ B p65/p50 DNA binding activity was not detected in the liver of untreated rats (lane 1) nor in the livers from non-hepatectomized rats 1 h after i.p. injection of saline (lane 2), PDTC (lane 4) or PTX (lane 6). NF- κ B

was strongly activated 0.5 h after PH preceded by saline injection (lane 3), similarly to activation observed 0.5 h after PH alone^[24]. Supershift analysis confirmed the binding of the heterodimer p65/p50 NF- κ B complex (lanes 8-10). Injection of PDTC or PTX 1 h before PH prevented the occurrence of the expected p65/p50 DNA binding complex at 0.5 h after PH, since the upper complex was not observed on EMSA (lanes 5 and 7). However, two distinct faster-migrating complexes have been found after PTX (lane 7). The first one, also present after saline or PDTC injection, was partially supershifted by p50 antibody; the second one totally disappeared with p65 antibody. Supershift assays performed with p52, rel B, and c-rel had no influence on this lower complex (Figure 1B).

Effect of PDTC and PTX on hepatocyte proliferation 24 h after PH (Figures 2 and 3)

Hepatocyte proliferation was followed by PH peaks at 24 h. Pretreatment of rats with PDTC or PTX 1 h prior to PH had no effect on liver regeneration. Thymidine incorporation was indeed similarly elevated in all the groups (Figure 2). In untreated rats, 90% of hepatocytes

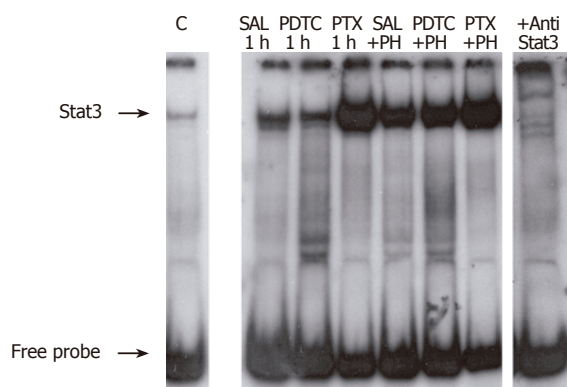


Figure 4 Effect of saline, PDTC and PTX on Stat3 DNA binding activity. No Stat3 DNA binding activity was identified in controls (C). Injection of saline, PDTC or PTX enhanced the binding at 1 h. At 0.5 h after PH, strong Stat3 DNA binding was observed in rats injected with saline, PDTC or PTX. Supershift experiments with a Stat3 specific antibody confirmed that Stat3 is the principal compound of these DNA-binding complexes.

nuclei expressed PCNA 24 h after HP. Pretreatment with PDTC or PTX did not modify the proportion of PCNA positive nuclei in agreement with the thymidine incorporation (Figure 3). No evidence of liver necrosis or massive apoptosis was observed by histological examination.

Effect of injection of saline, PDTC and PTX on Stat3 binding activity (Figure 4)

Stat3 DNA binding activity was not detected in the liver of untreated rats (lane 1), but a Stat3 DNA binding complex was present in the livers from non-hepatectomized rats receiving saline 1 h before being killed (lane 2), PDTC (lane 3) or PTX (lane 4). PH was associated with high Stat3 activity at 0.5 h in animals pretreated with saline (lane 5), PDTC (lane 6) or PTX (lane 7), contrasting with very low Stat3 activation after PH in non-injected rats at this time point. Indeed, as already reported by us and others, PH in non-injected rats was followed by slight Stat3 activation at 0.5 h after PH, peaked at 3 h and was still detected till 8 h^[24,26]. Identity of Stat3 was confirmed by supershift analysis with a specific Stat3 antibody (lane 8).

DISCUSSION

PH induces an early cellular response involving pro-inflammatory cytokines like TNF- α or IL-6^[27-29] and transcription factors molecules such as NF- κ B and Stat3^[3,4,30]. In the initiation of proliferating response following hepatic cell mass reduction, a key role seems to be attributed to the activation of the transcription factor NF- κ B. The inducible NF- κ B factor regulates the expression of numerous genes involved in immune, inflammatory and cellular growth control^[11,31,32]. Migrating to the nucleus as soon as 0.5 h after PH^[3], it induces the transcription of a large set of immediate-early genes^[33]. The NF- κ B p65 subunit, important for liver development^[34], is responsible for the transcriptional activity of NF- κ B^[35]

and also for its anti-apoptotic effects, preventing the cytotoxic effect of TNF- α and cell death^[36,37]. Convergent data suggest that this mediator collaborates with other acute phase gene products in order to protect the cells during the regenerative process^[5,38-40]. The most active form of NF- κ B is a heterodimer consisting of subunits p50 or NF- κ B1 and p65 also called RelA, which contains the transactivation domain necessary for the induction of target genes^[41,43]. This active form has been identified after PH as the post-hepatectomy factor^[3,30]. Although not consistently reported, the activation of NF- κ B after PH has been supposed to be related to an increased expression of TNF- α , although not consistently reported, and/or to oxidative stress^[2]. However, various other non specific stimuli are able to induce this activation^[44]. Although the importance of the TNF- α pathway has been outlined^[6-8], recent studies analyzing liver regeneration in different types of knockout mice suggest that a complete regenerative response may occur in the absence of early factors such as TNF- α , IL-6 or Stat3^[43,45]. In order to investigate the role of NF- κ B activation in determining the progression into the cell cycle and liver regeneration, we analyzed the effect of NF- κ B inhibition on hepatocyte proliferation after PH in normal rat with otherwise normal cytokine expression, regulation and signaling capabilities. Two strategies have been used to prevent NF- κ B activation. First, we administered an antioxidant molecule (PDTC) to reduce the oxidative stress, a known stimulus of NF- κ B activation. Second, we aimed at decreasing the influence of TNF- α by reducing its production by the use of PTX. PTX and PDTC effectively prevented the activation of NF- κ B p65/p50 complex observed 0.5 h after PH in non-treated and saline-treated animals. Despite this absence of NF- κ B activation, the hepatocytes responded to the proliferation stimulus: PCNA expression as well as DNA synthesis analyzed 24 h post-PH were normal compared to a classical PH. At that time we did not observe any evidence of liver necrosis or massive apoptosis compared to livers from untreated animals (data not shown). This last observation is in contrast with the results obtained when NF- κ B is inhibited by an adenoviral vector expressing a mutated form of I κ B- α ^[5]. The inhibition of NF- κ B using this adenoviral vector led to massive apoptosis but also failed to interfere with hepatocyte proliferation. This massive apoptosis could be induced by the adenovirus itself which causes increase of TNF levels and apoptosis before PH. More recently, it has been advocated that the activation of NF- κ B in hepatocytes is not needed to induce liver regeneration after PH^[10]. The authors also postulated that a preserved activation in non-parenchymal cells could be sufficient to drive both proliferative and anti-apoptotic effects of NF- κ B during liver regeneration. This last assumption cannot be found in our experiments, since the inhibition of NF- κ B was not targeted to a specific cell type in the liver and, in principal, the substances used in our experiments do act on all liver cell types including the non-parenchymal cells.

We observed that PTX induced another DNA binding

complex after PH, migrating faster, and partially supershifted by the p65 antibody. The functional role of such a complex is not elucidated but may constitute a compensatory response of the Rel family of transcription factors to substitute the usual factor. In a recent study using p50^{-/-} mice^[46], PH was associated with a normal regenerative response. P65 protein expression was elevated but without p65/p50 DNA binding activity suggesting that p65 could be part of other complexes, allowing normal regeneration. Considering our data and those obtained in p50 knockout mice, the p65/p50 DNA binding after PH does not seem to be absolutely required for proper liver regeneration. PTX is a potent inhibitor of TNF production which inhibited NF- κ B activation but without reducing liver regeneration. These data further reinforce the absence of a link between NF- κ B activation and hepatocyte proliferation. However, they do not bring any information concerning a role for TNF- α in the initiation of liver regeneration, since the expression of this factor was not investigated in the study. In contrast to the data demonstrating the quasi absence of Stat3 activation during the first hour after PH^[24,26], an early activation of Stat3 was observed as soon as 0.5 h after PH preceded by saline, PDTC and PTX. This suggested that Stat3 was already induced in our model by the first stimulus, i.e. the injection and the required animal handling. This hypothesis was confirmed by the increased Stat3 DNA binding obtained after a single injection of saline, PDTC and PTX. It seems therefore that Stat3 is a component of the acute-phase response induced by stress procedures as minimal as the i.p. injection. This rather non-specific origin for Stat3 activation does not exclude that this factor may play an important role in liver regeneration as documented in studies conducted in IL-6 and TNFR-1 knockout mice in which the restoration of the Stat3 DNA binding following IL-6 supplementation corrected the impaired liver regeneration process^[6,47]. It seems thus that Stat3 activation, which is a part of the priming process as described by Bucher *et al.*^[48], can be produced by a great variety of stimuli and not necessarily by the PH itself.

As demonstrated previously, sham operation also induced activation of NF- κ B and Stat3^[24]. PH preceded by a sham operation was associated with a second activation of these transcription factors^[49]. In this work, the i.p. injection is a minor stress compared to a sham operation. However, the i.p. injection procedure produced by itself an activation of Stat3 that persisted at the time of PH and probably for a longer period, possibly masking the effects induced by this second intervention. By contrast, a NF- κ B response was clearly observed after PH when only saline was injected. When drugs able to inhibit NF- κ B activation were injected, this response was indeed suppressed but the proliferation indices were not affected. An activation of liver NF- κ B seems thus unnecessary to initiate liver regeneration in those conditions. In conclusion, and in complement to those of others using a transgenic model, our data strongly suggest that an increase of liver NF- κ B p65/p50 DNA binding as an immediate response to PH is not essential for inducing liver cell proliferation.

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• RAPID COMMUNICATION •

Serum arylesterase and paraoxonase activity in patients with chronic hepatitis

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CONCLUSION: Low PON1 and AE activity may contribute to the increased liver dysfunction in chronic hepatitis patients by reducing the ability of HDL to retard LDL oxidation and might be clinically useful for monitoring the disease of chronic hepatitis.

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Key words: Paraoxonase; Arylesterase; Chronic hepatitis; Lipoproteins

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Abstract

AIM: To investigate the relationship between serum paraoxonase (PON1), AST, ALT, GGT, and arylesterase (AE) activity alterations and the degree of liver damage in patients with chronic hepatitis.

METHODS: We studied 34 chronic hepatitis patients and 32 control subjects, aged between 35 and 65 years, in the Department of Infection and Clinical Microbiology at the Firat University School of Medicine. Blood samples were collected from subjects between 8:00 and 10:00 a.m. following a 12-h fast. Baseline and salt-stimulated PON1 activities were measured by the hydrolysis of paraoxon. Phenyl acetate was used as the substrate and formed phenol was measured spectrophotometrically at 270 nm after the addition of a 10-fold diluted serum sample in AE activity measurements.

RESULTS: The results of this investigation revealed that the levels of AE activity decreased from 132 ± 52 to 94 ± 36 (29%), baseline PON1 activity from 452 ± 112 to 164 ± 67 (64%), salt-stimulated PON1 activity from 746 ± 394 to 294 ± 220 (61%), HDL from 58.4 ± 5.1 to 47.2 ± 5.6 (20%), triglyceride from 133 ± 51.2 to 86 ± 34.0 (35%), while a slight increase in the level of LDL (from 163 ± 54.1 to 177.3 ± 56.0 ; 9%) and significant increases in the levels of AST (from 29 ± 9.3 to 98 ± 44), ALP (from 57.2 ± 13.1 to 91 ± 38.1), ALT (from 27.9 ± 3.32 to 89 ± 19.1), GGT (from 24.3 ± 2.10 to 94 ± 48.2), total bilirubin (from 0.74 ± 0.02 to 1.36 ± 0.06 ; 84%) and direct bilirubin (from 0.18 ± 0.01 to 0.42 ± 0.04 ; 133%) were detected. However, the levels of albumin, total protein, cholesterol, and uric acid were almost the same in chronic hepatitis and the control subjects.

INTRODUCTION

The paraoxonase (PON1), a 44-ku glycoprotein, is synthesized mainly in the liver, which hydrolyzes organophosphates like pesticides, neurotoxins, and arylesters^[1-4]. PON1 activity exhibits a substrate-dependent activity polymorphism in human beings^[4-6]. This polymorphism is related to two polymorphic sites at amino acid positions. One at position 192, which is a glutamine→arginine substitution, hydrolyzes paraoxon with a high enzyme activity, and the other at position 55, which is a leucine→methionine substitution causes a low enzyme activity^[7]. The activity of PON1 shows great inter-ethnic variability^[8-11]. The gene frequency for PON1_{R192} allele is 0.31 in Turkish population^[11] and in Caucasian population^[8], 0.41 in Hispanic populations, 0.66 in a Japanese population^[8]. Three different phenotypes are reported based on the responses of the two isoenzymes to salt concentrations^[6,12]. The ratio of salt-stimulated PON1 activity to arylesterase (AE) activity is used for the definition of phenotypes^[6,12,13].

Environmental factors that change PON1 activity include tobacco consumption, which has been reported to depress PON1 activity and concentration^[14]. Also, several studies have shown that PON1 level is tightly linked with HDL in the serum and contributes to the protection conferred by HDL against LDL oxidation. Human paraoxonase (PON), an HDL-associated enzyme carried on apo A-I, is believed to protect lipoproteins against oxidative modification^[15,16].

As mentioned above, the role of PON1 and AE activity may be particularly meaningful as an index of liver func-

tion status because preliminary studies have revealed a remarkable decrease of serum AE activity in patients with liver cirrhosis^[17,18], and AE and PON1 activities have been demonstrated to be a function of a single enzyme^[19].

To the best of our knowledge, although AE and PON1 activities have been demonstrated to be a function of a single enzyme^[19], both PON1 (only studied by Ferre *et al.*^[20]), and AE activity (only studied partly^[17,18]) in chronic HBV have not been studied together, as yet. The paucity of data on serum PON1 and AE does not allow for a conclusion about its role in chronic HBV. Therefore, we carried out this study aiming to compare PON1 and AE activities and traditional standard biochemical test of liver function parameters (such as, ALT, AST, and so on), and whether there were changes due to chronic hepatitis.

MATERIALS AND METHODS

We studied 34 chronic hepatitis patients and 32 control subjects, aged between 35 and 65 years, in the Department of Infection and Clinical Microbiology at the Firat University School of Medicine. Control subjects were healthy and evaluated for any symptoms of clinical or analytical evidence of diabetes, renal disease, cardiovascular disease, neoplasia, or hepatic damage and were matched for age and body mass index. Cirrhosis was graded according to Child-Pugh criteria^[21]. Blood samples were collected from subjects between 8:00 and 10:00 a.m. following a 12-h fast. The extracted blood samples were then centrifuged at 4 000 r/min for 10 min and stored at -70 °C until the assay was performed. This study was performed with the approval of the ethics committee and all subjects volunteered for the study with informed consent.

Assay of paraoxonase activity

PON1 assays were done without additional NaCl (baseline activity) and with 1 mol/L NaCl included in the assay buffer (salt-stimulated activity), following the formation of *p*-nitrophenol by its absorbance at 405 nm for 5 min. Assay buffer contained 0.125 mol/L Tris-HCl (pH 8.5), 1.25 mmol/L CaCl₂ and 1 mol/L NaCl (pH 8.5)^[4,12]. For each set of assays, 6 mmol/L freshly prepared paraoxon (*O,O*-diethyl-*O-p*-nitrophenylphosphate; Sigma Chemical Co.) substrate solution of 120 mmol/L paraoxon in acetone diluted with 0.125 mmol/L Tris-HCl was used. Paraoxon stock solution was handled very cautiously with protective measures. The assay tube contained 750 µL of Tris buffer, 50 µL of serum (1:2 diluted with water) and 200 µL of 6 mmol/L paraoxon. The reaction was initiated at 37 °C by the addition of the substrate solution, and using a Techcomp 8500 II Spectrophotometer, absorbance was continuously monitored at 405 nm and 25 °C. The PON1 unit was defined as the enzyme quantity that disintegrates 1 µmol paraoxon substrate in one minute^[4,12]. The percent stimulation of PON1 was calculated as follows^[6]: [(PON1 activity with 1 mol/L NaCl)-(basal activity)/basal PON1 activity]×100.

Assay of arylesterase activity

AE activity was measured with phenylacetate as a substrate as previously described^[17,18,22]. AE activity was affected with salt. The assay tube contained 750 µL of 0.1 mol/L Tris-HCl (pH 8.5), 1 mmol/L CaCl₂, 125 µL of 12 mmol/L phenylacetate and 125 µL of serum (1:10 diluted with water). The absorbance was continuously monitored at 270 nm and 37 °C. The units were expressed as millimoles of phenylacetate hydrolyzed per minute.

Biochemical parameters of liver function

Serum AST, ALT, ALP, GGT, albumin, total protein, indirect bilirubin, total bilirubin and other biochemical parameters were analyzed with an auto analyzer (Olympus 600).

Statistical analysis

Statistical analysis was performed using Student's *t* test for group comparisons and data for biochemical analyses were expressed as mean±SD. Pearson's correlation coefficients were used to test the correlation between each of the two biochemical variables. Multiple linear regression analysis was used between PON1 and AE activity and possible determinants, such as AST, ALT, ALP, and GGT. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Laboratory results of the standard liver functions tests are documented in Table 1, and provide evidence of the spectrum of disease. As expected, the levels of AST, ALT, ALP, and GGT in the chronic hepatitis patients were significantly increased as compared to the controls. Increased values of transaminase were also matched by a corresponding rise in serum bilirubin. The serum PON1 and AE were obviously correlated with each HDL parameter and triglyceride (TG) in chronic hepatitis patients compared to the controls.

In the present study, we also observed markedly elevated LDL (9%) and decreased HDL (20%) in chronic hepatitis patients as compared to the controls (Table 1). The activities of AE were significantly reduced (by 29%) in the chronic hepatitis group (94±36 U/mL) compared with the control group (132±52 U/mL, *P*<0.001, Table 1). Furthermore, PON1 baseline activity was significantly decreased (by 64%) in the chronic hepatitis group (164±98 U/mL) as compared with the control group (452±138 U/mL, *P*<0.001, Table 1). Also, salt-stimulated serum PON1 activity was decreased (by 61%) in chronic hepatitis group (294±220 U/mL) as compared with the control group (746±394 U/mL, *P*<0.001, Table 1).

Comparison of serum PON1/AE activity and traditional standard biochemical test of liver function

We observed negative correlations between ALT and AE activities (*r* = -0.112, *P*<0.11), as well as between the percent stimulation of PON1 and ALT (*r* = -0.412, *P*<0.05) in the chronic hepatitis patients, but not in the controls (*r* = 0.142). In addition, we found obvious negative

Table 1 Traditional standard biochemical test of liver function and some other parameters (mean±SD)

Parameters	Control (n = 32)	Chronic hepatitis (n = 34)
ALT (U/L)	27.9±3.12	89±19.1 ^b
AST (U/L)	8±4.4	29±9.3
GGT (U/L)	24.3±2.10	94±48.2 ^b
ALP (U/L)	57.2±13.1	91±38.1 ^b
Albumin (mg/dL)	4.83±0.28	4.62±0.17
Total bilirubin (mg/dL)	0.74±0.02	1.36±0.06 ^b
Direct bilirubin (mg/dL)	0.18±0.01	0.42±0.03 ^b
Total protein (mg/dL)	7.3±0.18	7.3±0.42 ^a
Cholesterol (mg/dL)	196±23.3	187±23.2 ^a
Uric acid (mg/dL)	4.9±0.33	5.2±0.29 ^a
HDL (mg/dL)	58.4±5.4	47.1±5.6 ^a
LDL (mg/dL)	163±54.1	177.3±56.0 ^b
TG (mg/dL)	133±51.2	86±34.0 ^b
¹ Salt-stimulated PON1 (U/mL)	746±364	294±220 ^a
Baseline PON1 (U/mL)	452±112	164±67 ^b
AE (U/mL)	132±52	94±36 ^b

^aP<0.05, ^bP<0.001 *vs* control subjects; ¹salt stimulated activity.

correlations between GGT and AE activities ($r = -0.901$, $P < 0.01$), as well as between the percent stimulation of PON1 and ALT ($r = -0.412$, $P < 0.05$), but not such correlation in the controls ($r = -0.122$, not significant). We also previously reported similar correlations between AE and PON levels in severe pre-eclamptic women^[23].

ALP activity was also correlated with PON1 ($r = -0.41$, $P < 0.05$) and AE ($r = -0.44$, $P < 0.03$) activities in the chronic hepatitis group, but not in the controls ($r = 0.102$). This moderate correlation might be a result of ALP demonstration in different tissues such as bone and small bowel when compared with ALT, which is demonstrated only in the liver.

DISCUSSION

Chronic HBV infection is one of the most important diseases leading to a high morbidity and mortality due to the development of liver failure, liver cirrhosis (LC), and liver cancer^[24,25]. There are over 300 million people suffering from HBV infection worldwide^[26]. The destruction of liver cells can be extensive, and death follows from liver failure in about 1% of cases. Many of those recover progress to shed the virus for years, while some develop a progressive degenerative liver disease called chronic active hepatitis^[24,25,27]. Dramatic alterations in chronic hepatitis remain within laboratory values until gross disease becomes evident. To diagnose such a slow progressive liver disease (chronic hepatitis) before advancing hepatocellular necrosis and fibrosis, beside traditional biochemical tests needs alternative parameters to evaluate liver damage^[20].

PON1 and AE activities have been demonstrated in different tissues, such as liver (including microsomes), kidney^[28,29], brain^[30,31] and lung^[30], and it has been studied extensively in relation to cardiovascular diseases^[2,3,5,16], whereas there are scarce data available on the hepatic

enzyme. Some of these enzymes are released into the circulation and some portions are stored in the liver. Serum PON1, which is carried in circulation bound to HDL particles, protects LDL from peroxidation^[2,3,10,14-16,32]. The putative function of PON1 in the liver is to provide hepatic protection against oxidative stress^[33].

In the present study, the relation between serum PON1 and AE activity levels and chronic hepatitis was examined. We observed that PON1 and AE activities were obviously lower in the chronic hepatitis patients compared with the control subjects, and were significantly correlated with each HDL parameter, and consistent with reports that this enzyme is bound to a large apo A-I containing HDL subspecies and also confirmed previously reported results^[10,15,34-36]. The decrease in PON1/AE activity in the serum was also correlated with serum AST, ALT, GGT, albumin, and bilirubin concentrations. It has been reported that the diagnostic accuracy of PON1 is equivalent to that of ALT in patients with chronic hepatitis and far superior to that of the other tests in patients with cirrhosis^[20]. Our results are in agreement with some recent studies in cirrhotic patients^[20], rats^[33], and chronic hepatitis patients^[17,18].

Serum PON1 and AE are mainly the result of liver activity^[17]. We have some possible explanations for decreased PON1 and AE activities with chronic hepatitis. One is that even though hepatic PON1 and AE levels may be normal, serum PON1 and AE activity would be lowered as a result of changes in synthesis or secretion of the HDL secondary. This assumption is supported by other researchers^[20,37-39]. Alterations in HDL structure and levels related to decreased serum PON1 activities in mice with LCAT deficiency are the consequence of LCAT gene-targeted disruptions. PON1 and AE have also been reported to play a role in the lipid transfer and assembly of VLDL particles in liver microsomes^[39]. The other is that liver cells damaged via chronic hepatitis do not express the protein PON1 and AE. Both have been shown to be functions of single enzymes. Supporting this putative function is the report of inhibition of microsomal PON1 activity in rats with chronically administered CCl₄^[32]. So, elevated LDL and decreased HDL might have a causal role in the pathogenesis of chronic hepatitis. It is known that PON1 is bound to HDL and acts as an antioxidant that protects LDL from oxidative modifications and can reduce oxidized lipids in oxidized lipoproteins^[23].

In conclusion, a significant decrement in PON1 and AE is probably the consequence of liver dysfunction. For this reason, serum PON1 and AE activities could be a beneficial tool for chronic hepatitis patients. However, further research is needed to elucidate the mechanism leading to decreased serum PON1 and AE activities in liver diseases and potential pathophysiologic implications.

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• RAPID COMMUNICATION •

Effects of fermented soy milk on the liver lipids under oxidative stress

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Abstract

AIM: To investigate the effects of fermented soy milk powder on the antioxidative status and lipid metabolism in the livers of CCl₄-injected rats.

METHODS: Forty-five healthy male Sprague-Dawley rats were randomly assigned to five groups according to five different diets: control (AIN-76), AIN-76+high-dose fermented soy milk powder, AIN-76+low-dose fermented soy milk powder, AIN-76+high-dose milk yogurt powder and AIN-76+low-dose milk yogurt powder. The experiment lasted for 8 wk. After 4 wk, all the rats received intraperitoneal administration of CCl₄ (0.2 mL/100 g body weight) every week. Total cholesterol (TC), triglyceride (TG), TBARS, ALP, and antioxidative enzymes in the liver were evaluated.

RESULTS: There was also no significant difference in TBARS and antioxidative enzymes in the liver. TC and TG in the groups fed with fermented soy milk powder were generally lower than those fed with casein powder.

CONCLUSION: Consumption of fermented soy milk was positive in lowering total cholesterol and TG accumulation in the liver under CCl₄-induced oxidative stress.

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Key words: Soy; Fermented soy milk; Antioxidative; Liver protection

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INTRODUCTION

Soybeans contain valuable nutritional attributes. It has been found that the intake of soy foods is closely related to lowering the occurrences of chronic diseases^[1]. There are many functional ingredients contained in soy foods such as soy protein, isoflavones, saponins, phytic acid, phytosterol, and phenolic acid^[2-4]. Compared to casein, soy protein showed a greater antioxidative ability in preventing lipid oxidation^[5]. Isoflavones have been found to increase the activities of some antioxidative enzymes in the liver^[6]. Soy foods in Oriental countries can be divided into two categories: unfermented and fermented products. Fermented soy products have been greatly researched recently because their nutritional attributes may be changed due to the metabolism of microorganisms. Fermented soy milk, unlike fermented milk or yogurt drinks, contains no lactose or cholesterol and may have the health benefits from both soy itself and the fermentation. Cheng *et al.*^[7] suggested that consumption of fermented soy milk was beneficial to human intestinal health. Our purpose was to investigate the effect of fermented soy milk on liver protection under induced oxidative stress.

MATERIALS AND METHODS

Fermented soy milk

Fermented soy milk was provided by Taiwan Tobacco & Liquor Cooperation (TTL, Taipei, Taiwan, China) Spray drying was applied to produce fermented soy milk powder.

Animals

Forty-five 6-wk-old healthy male Sprague-Dawley (SD) rats (National Laboratory Animal Center, Taiwan, China) were randomly assigned to five groups according to different diets. Guidelines for the ethical care and treatment of animals from the Animal Care Committee at Taipei Medical University were strictly followed. Rats were individually housed and maintained in a temperature-controlled (23±2 °C) room with a 12-h light/dark cycle. They were fed a chow diet for 1 wk before switching to the experimental diet. Five rats were killed to acquire the baseline biological values of liver. Water and food were available *ad libitum*.

Treatment and sample collection

Fasting blood samples from the tail vein were collected in tubes containing heparin on the last day of wk 0, 2, 4, and 6.

Table 1 Composition of different diets (g/25 g)¹

	CC ²	HCS	LCS	HCM	LCM
Corn starch	13.2	4.8	9.0	6.7	9.8
Casein	5.0	4.4	4.7	2.1	4.0
Cellulose	1.6	1.1	1.4	1.1	1.4
Soybean oil	1.5	1.5	1.5	1.5	1.5
Mineral mixture	1.5	0.9	1.2	0.9	1.2
Sucrose	1.5	0.9	1.2	0.9	1.2
Vitamin mixture	0.5	0.3	0.4	0.3	0.4
Methionine	0.1	0.1	0.1	0.1	0.1
Fermented soy milk		10.0	5.0		
Milk yogurt				10.0	5.0

¹Diets were iso-caloric; ²CC, control group; HCS, high-dose fermented soy milk powder; LCS, low-dose fermented soy milk powder; HCM, high-dose fermented milk powder; LCM, low-dose fermented milk powder.

The blood was then centrifuged for 15 min at 1 400 r/min to separate the plasma and erythrocytes and stored at -80 °C. Starting from the 4th wk, rats received an abdominal injection of CCl₄ (0.2 mL/100 g body weight) once a week until the end of the experiment. The body weight of each rat was recorded every 2 d. At the end of wk 8, rats were killed, and liver samples were collected, weighed, and stored at -80 °C.

Diets

The diets were iso-energetically formulated (Table 1). The control diet was AIN76 (CC). Four experimental diets were prepared by mixing 10 g (HCS), 5 g of fermented soy milk powder (LCS), 10 g (HCM), and 5 g of milk yogurt powder (LCM).

Preparation of liver homogenates and lipid extraction

For preparing liver homogenate, 1.5 g of liver samples was mixed with 2.5 mL of buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl, pH 7.4) and blended at 4 °C (avoiding light), followed by centrifugation at 4 500 r/min for 6 min. The isolated liver cells were stored at -80 °C.

For extracting liver lipid, 1 g of the liver samples was mixed well with 12 mL of chloroform/methanol (2:1) and then filtered. The filtrate was evenly distributed into two test tubes and chloroform/methanol/H₂O (3:48:47) was added to each tube to 10 mL. Then 2 mL of 0.05% CaCl₂ was added to each tube, followed by degassing for 15 min and centrifugation at 2 500 r/min and 4 °C for 3 min. The supernatant was removed and methanol was added to the sample to 10 mL. The sample in each tube was then mixed together, to which chloroform/methanol (2:1) was added to 25 mL.

Alkaline phosphatase (ALP) determinations

ALP was evaluated using commercial kits (ALP reagent L-type, Wako).

Activities of antioxidant enzymes in the liver

The activities of antioxidative enzymes were determined using commercial kits: superoxide dismutase (SOD, Calbiochem), glutathione peroxidase (GPx, Randox), and glutathione reductase (GR, Randox).

Table 2 Body weights of each group fed with different diets (mean±SD, g)

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
	(Before injection of CCl ₄)		(After injection of CCl ₄)		
CC ¹	223.6±13.61	267.4±16.51	317.0±30.2	371.9±36.5	402.1±25.2
HCS	237.7±11.81	276.5±11.11	338.7±14.11	395.3±17.91	429.0±27.01
LCS	231.4±14.31	272.1±11.01	329.2±9.41	395.0±17.91	435.4±26.71
HCM	231.6±9.71	264.7±9.41	314.3±11.0	367.4±12.82	403.1±10.3
LCM	225.2±10.8	260.6±14.3	309.9±19.9	364.5±17.02	405.3±17.4

¹CC, HCS, LCS, HCM, LCM, as in Table 1. *n* = 7-9.

Table 3 Plasma ALP concentration (IU/dL) of each group fed with different diets¹

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
	(Before injection of CCl ₄)		(After injection of CCl ₄)		
CC	33.8±0.0	51.3±9.5	43.8±14.0	194.4±65.6	303.7±119.9
HCS	50.2±11.1	70.5±8.9	57.8±6.6	472.6±235.1	338.6±130.3
LCS	49.9±21.3	64.2±4.4	47.1±3.6	278.6±205.7	360.7±206.1
HCM	35.1±6.9	56.4±4.9	39.2±6.7	577.8±74.2	548.0±150.8
LCM	37.7±6.8	57.3±8.0	43.0±3.5	255.1±170.3	592.8±115.6

Thiobarbituric acid reactive substances (TBARS)

A blank (0.1 mL of water), a standard (0.1 mL of 4.2 μmol/L malonaldehyde), or 0.1 mL of the sample was mixed well with the reagent (a mixture of 4 mL of 22% sulfuric acid, 0.5 mL of 10% phosphotungstic acid, and 1 mL of 0.67% 2-thiobarbituric acid). Then the mixture was heated in a water bath to 95 °C for 1 h. After being cooled with ice water, 2 mL of butanol was added to the sample, followed by centrifugation at 3 000 r/min for 15 min. The optical density of the pinkish upper part was determined using a fluorescence spectrometer with excitation at 515 nm and emission at 555 nm.

Statistical analysis

All the results were presented as mean±SD. Two-way analysis of variance (ANOVA) and the least significant difference test were performed using SAS[®] 6.13 to analyze the time and diet effects.

RESULTS

Body weights of the animals

During the feeding period, body weights in each group of all the rats increased (Table 2). At the end of the feeding period, the body weights of rats fed with fermented soy milk powder (HCS and LCS) were significantly higher than those in the other groups (*P*<0.05). None of the rats showed any abnormal condition.

Alkaline phosphatase (ALP) determinations

ALP activity in the plasma was used to evaluate the liver function. Before injection (i.e. before wk 4) of CCl₄, the ALP (Table 3) in any group did not significantly differ. After injecting CCl₄, ALP activities in all the groups increased (*P*<0.05). HCS and HCM had higher ALP activities than did the other groups. After wk 6, the ALP activities in the HCS and HCM groups had significantly decreased (*P*<0.05). In wk 8, the ALP activity in the HCS

Table 4 Activities of antioxidative enzymes and concentrations of TBARS, TC, and TG of the liver in CCl₄-treated rats¹ (mean±SD)

	Baseline	CC ¹	HCS	LCS	HCM	LCM
CAT ²	0.92±0.19	0.25±0.14	0.30±0.123	0.45±0.25	0.47±0.16	0.44±0.27
GPx	703±154	615±166	680±141	857±179	734±94	762±120
GR	613±288	591±160	768±94	906.4±158	772±283	668±125
SOD	0.118±0.035	0.094±0.015	0.082±0.007	0.088±0.026	0.111±0.035	0.084±0.024
TBARS	6.32±0.71	5.53±1.00	6.08±0.77	4.79±0.67	6.24±0.97	5.92±0.97
TC	5.54±0.73	6.69±2.77	5.37±2.71	4.16±1.55	7.07±2.83	7.06±2.85
TG	12.82±0.69	15.86±3.93	18.633±5.62	16.76±0.067	19.60±0.79	20.62±5.21

group had decreased even lower than those in the HCM group ($P<0.05$).

Antioxidative enzymes, TBARS, TC, and TG in the liver (Table 4)

Results showed that at the end of the feeding period, the catalase activity in the liver in all the groups decreased ($P<0.05$), while the activities in CC and HCS rats were significantly lower than those in the other groups ($P<0.05$). The activity of GPx in all the groups did not significantly change through the experiment. The activity of GR in the LCS group significantly increased at the end and was higher than those in the other groups ($P<0.05$), while those in the other groups did not differ from each other and from the baseline. The activities of SOD in all the groups decreased at the end but only those in the HCS and LCS groups were significantly lower than that in the baseline ($P<0.05$).

At the end of feeding period, the TABRS in all the groups did not differ from each other but tended to be lower or significantly lower (CC, LCS, and LCM; $P<0.05$) than that in the baseline. Rats in the LCS and HCS groups had lower TC value than did those in the HCM, LCM, and control groups ($P<0.05$). Rats in the LCS and HCS groups tended to have lower TG than did those in the HCM and LCM groups.

DISCUSSION

The isoflavone contents of HCS and LCS groups were 0.97 and 0.47 mg/d, respectively in our experiment. Converting the amounts for rats to that for a 70-kg adult, values were about 54 and 27 mg of isoflavones daily although the isoflavone contents in the plasma were either trace or undetectable. It has been pointed out that a daily intake of 50 mg of isoflavones is beneficial to health^[8]. The reason why the isoflavones were not detected was due to that all rats were fasted for 8 h before their blood samples were collected. It was found that daidzein and genistein in the plasma were undetectable in rats after 8 h of ingestion^[9-11]. The time might have been too long for the isoflavones to be detected.

We found that under such doses of CCl₄, all rats in this experiment had similar activities of GPx and GR in the liver no matter if they had consumed fermented soy milk powder or not. This could be due to a relatively high dose of CCl₄. Zavate^[12] pointed out that injecting a dose of 0.2 g/100 g BW of CCl₄ in rats resulted in decreases or deactivation in the activities of catalase, SOD, and GPx,

and increases in lipid oxidation in the liver. In addition, the CCl₄ injection produced acute damage to the liver. Protection of the liver through food consumption may not be effective enough in a short period.

In the diets of HCS and LCS groups, we found 3-hydroxyanthranilic acid (3-HAA; data not shown). 3-HAA is a by-product of soy fermentation^[13]. It has been found as effective as α -tocopherol and was more effective than genistein^[14,15] in inhibiting lipid oxidation *in vivo*. The effect of 3-HAA on protecting liver lipids from oxidation needs further research. Other than 3-HAA, many components in soy are antioxidative. Soy isoflavones, soy protein, and saponins all possess antioxidative abilities^[16]. Our data did not clearly suggest that consumption of fermented soy powder was effective in reducing TBARS. This could be due to the limitation when applying TBARS to evaluate lipid oxidation in the liver. Massie *et al.*^[17] pointed out that the storage time of liver samples before measuring TBARS affected the results. In addition, the antioxidants such as BHT and EDTA added to the test tubes during the preparation of the liver homogenate also affected the outcomes^[18]. In our experiment, addition of EDTA was included in the procedure. In addition, Morrow *et al.*^[19] found that under high oxidative stress, the oxidative damages to the liver resulted in an increase in F2-isoprostane (F2-IsoPs) which could not be evaluated with TBARS method.

Our result suggested that consumption of fermented soy milk powder slightly reduced the accumulation of TG in the liver caused by CCl₄. Pencil *et al.*^[20] indicated that CCl₄ inhibited the secretion of lipoproteins in the liver and altered the metabolism of fatty acids and resulted in fatty livers.

In conclusion, consumption of fermented soy milk was positive in lowering total cholesterol and TG accumulation in the liver under oxidative stress. Fermented soy milk drink is relatively new to the market. The results open an opportunity to further research on the amount and formula of fermented soy milk needed to achieve health benefits.

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• RAPID COMMUNICATION •

Alteration of chaperonin60 and pancreatic enzyme in pancreatic acinar cell under pathological condition

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Abstract

AIM: To investigate the changes of chaperonin60 (Cpn60) and pancreatic enzymes in pancreatic acinar cells, and to explore their roles in the development of experimental diabetes and acute pancreatitis (AP).

METHODS: Two different pathological models were replicated in Sprague-Dawley rats: streptozotocin-induced diabetes and sodium deoxycholate-induced AP. The contents of Cpn60 and pancreatic enzymes in different compartments of the acinar cells were measured by quantitative immunocytochemistry.

RESULTS: The levels of Cpn60 significantly increased in diabetes, but decreased in AP, especially in the zymogen granules of the pancreatic acinar cells. The elevation of Cpn60 was accompanied with the increased levels of pancreatic lipase and chymotrypsinogen in diabetes. However, a decreased Cpn60 level was accompanied by high levels of lipase and chymotrypsinogen in AP. The amylase level was markedly reduced in both the pathological conditions.

CONCLUSION: The equilibrium between Cpn60 and pancreatic enzymes in the acinar cells breaks in AP, and Cpn60 content decreases, suggesting an insufficient chaperone capacity. This may promote the aggregation and autoactivation of the premature enzymes in the pancreatic acinar cells and play roles in the development of AP.

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Key words: Chaperonin60; Pancreatic enzymes; Immunocytochemistry; Diabetes; Acute pancreatitis

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INTRODUCTION

Recently, a great deal of work has focused on a family of specific factors known as molecular chaperones. It has been established that molecular chaperones are involved in protein folding, assembly, disassembly, and degradation under normal conditions. It has also been established that molecular chaperones respond to stress states and certain disorders^[1-4]. Previous studies demonstrated that Cpn60 was co-distributed with various secretory proteins in acinar cells of the mammalian exocrine pancreas and associated with pancreatic secretory proteins in the intracellular compartments involved in the secretion, such as the rough endoplasmic reticulum (RER), the Golgi (G), and the zymogen secretory granules (ZG). Cpn60 follows the RER → G → ZG pathway in an increasing gradient as pancreatic enzymes^[1,5,6]. The association of Cpn60 and pancreatic enzymes along the secretory pathway was confirmed by immunoprecipitation assay on isolated zymogen granule extracts, where amylase and lipase co-precipitated with Cpn60^[5]. However, to our knowledge, there is little information available in literature about the relationship between Cpn60 and the enzymes change and also their significance in pancreatic pathogenesis. Therefore, streptozotocin-induced diabetes and deoxycholate-induced acute pancreatitis (AP) models in the Sprague-Dawley (SD) rats were replicated in order to explore the possible alterations of Cpn60 and pancreatic enzymes in the pancreatic acinar cells under the two experimental pathological conditions.

MATERIALS AND METHODS

Chemicals

Rabbit anti-Cpn60 was purchased from Stressgen, Victoria, BC, Canada. Rabbit anti-lipase, rabbit anti-chymotrypsinogen and protein A-gold complex were prepared in the laboratory as previously described^[7,8]. Rabbit anti-amylase, sodium deoxycholate (NaDc), urethane and some other chemical reagents were purchased from Sigma Chemical Co., St. Louis, USA.

Animals and preparation of pathological models

SD male rats ($n = 15$, part from St. Charles River, Quebec, Canada and part from the Animal Center, Shanghai Medical College of Fudan University, China) were randomly divided into three groups: normal control group ($n = 4$), AP group ($n = 6$) and diabetes group ($n = 5$). (1) AP model: Six rats (each weighing 250 g) were fasted overnight with free access to water. They were anesthetized by an intraperitoneal injection of urethane at 1 g/kg body weight. AP was induced by a retrograde injection of 4% NaDc into the biliopancreatic duct (BPD) using a modified method of Zhang *et al.*^[9,10]. After a small median laparotomy, the BPD was temporarily closed at the liver hilum with a soft microvascular clamp to prevent reflux of the infused material into the liver. A retrograde injection of 4% NaDc into the distal BPD was performed (40 mg/kg body weight). The clamp was removed 5 min after the injection. The abdomen was closed and the rats were then kept for 5 h. (2) Diabetes model: Five rats were administered with a single intraperitoneal injection of streptozotocin dissolved in 10 mmol/L citrate buffer (pH 4.5), at 70 mg/kg body weight. The hyperglycemic state developed in the first 72 h after injection and remained throughout the 3 mo of the experiment.

Tissue processing

At the end of the experiments, all the rats were anesthetized with urethane and the splenic parts of the pancreases were removed and fixed by immersion with 10 g/L glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at 4 °C. The tissue samples were washed with the phosphate buffer, dehydrated in a series of graded ethanol solutions, and embedded in Lowicryl K4M at -20 °C as described previously^[7]. Ultrathin sections were cut, mounted on Parlodion-carbon-coated nickel grids, and processed for immunocytochemistry.

Immunocytochemistry

The ultrathin sections mounted on nickel grids were incubated by floating them on a drop of 1% ovalbumin in PBS (pH 7.2) for 10 min at room temperature (RT). They were then transferred separately to a drop of differently diluted primary antibodies for 2 h at RT (except 4 h for anti-amylase). The antibodies used here included rabbit anti-Cpn60 (1:10), rabbit anti-lipase (1:10), rabbit anti-chymotrypsinogen (1:10), and rabbit anti-amylase (1:500). The grids were rinsed with PBS, transferred to the ovalbumin solution for 10 min, and then incubated on a drop of the protein A-gold complex (10 nm in diameter)^[6] for 30 min. They were then thoroughly washed with PBS and rinsed with distilled water. After staining with uranyl acetate, the sections were examined under a Philips 410 electron microscope. The specificity of each immunolabeling was tested effectively by two control experiments described previously^[7].

Evaluation

Morphometrical evaluation of the labeling densities, as a reflection of the quantity, was performed using

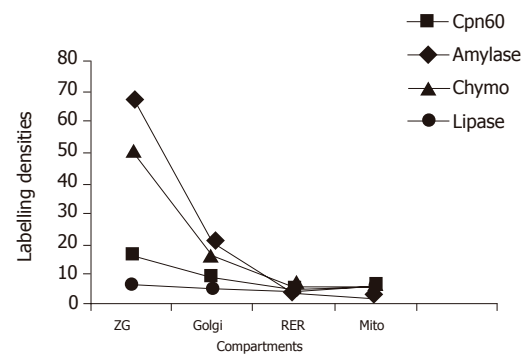


Figure 1 Distribution of Cpn60 and pancreatic enzymes in the compartments of the pancreatic acinar cells in normal rats. Labeling densities (gold particles/ μm^2) were measured by immunocytochemistry. ZG: zymogen granules; RER: rough endoplasmic reticulum; Mito: mitochondria; Chymo: chymotrypsinogen.

a Videoplan 2 image processing system. At least 30 micrographs, recorded at 28 000 \times final magnification, were analyzed for each animal tissue. Labeling densities were evaluated as described previously^[6] and were reported as the mean of gold particles/ $\mu\text{m}^2 \pm \text{SE}$ to the mean. Statistical analyses of the results were carried out using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Distribution of Cpn60 and pancreatic enzymes in the pancreatic acinar cells of rats

Immunocytochemistry labeling study on the sections of the pancreatic tissue revealed that in the acinar cells, both Cpn60 and the pancreatic enzymes, such as lipase, chymotrypsinogen, and amylase, co-existed in the RER, Golgi, and ZG. Increasing gradients of the labeling densities for both Cpn60 and the enzymes were present along the RER-G-ZG secretory pathway, with very low labeling in the mitochondria, in the normal rats and also in the experimental groups. (Figure 1).

Changes of Cpn60 and pancreatic enzymes in the pancreatic acinar cells of diabetic rats

Compared with the normal group, the sections from the diabetic rats showed a significant increase in the Cpn60 labeling density, and also in the lipase and chymotrypsinogen labeling densities, especially in the zymogen granules of the pancreatic acinar cells (Figures 2 and 3). In addition, the immunolabelings of Cpn60 increased nearly 3-fold, lipase 2.5-fold, and chymotrypsinogen was twice as high as the normal group ($P < 0.05$), but amylase level decreased drastically to a very low level ($P < 0.01$ vs normal group, Figure 3).

Changes of Cpn60 and pancreatic enzymes in the pancreatic acinar cells of AP rats

In AP, however, the labeling densities of Cpn60 declined through the secretory pathway (RER-G-ZG) of the

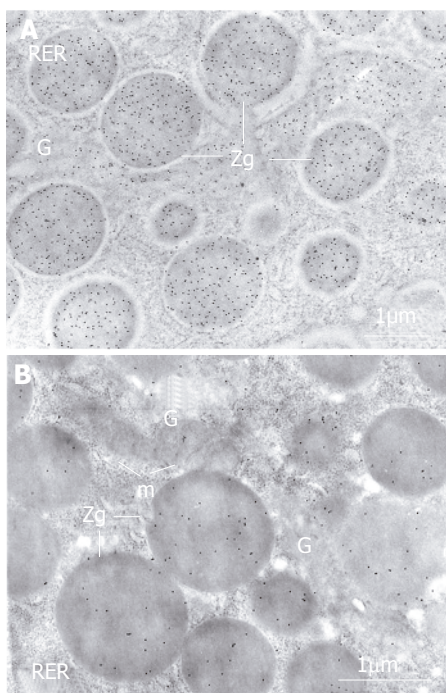


Figure 2 Changes of Cpn60 and pancreatic enzymes in the acinar cells of diabetic rats. The labeling by gold particles (black particles), reflecting Cpn60 antigenic sites, is more intense over the zymogen granules (ZG) in the pancreatic acinar cell in diabetic rat (A) compared with that in normal rat (B). RER: rough endoplasmic reticulum; G: Golgi; m: mitochondria.

pancreatic acinar cells (Figure 3), more significant in the zymogen granules where Cpn60 content decreased about 40% as compared with the normal group ($P < 0.05$). However, lipase markedly increased and chymotrypsinogen was kept at a high level in the compartment, suggesting a nonparallel change with Cpn60 (Figure 3).

DISCUSSION

Molecular chaperones, including chaperonins (Cpn60 and Cpn10), heat shock proteins (Hsps) and other components are a large family of specialized proteins present in various cellular compartments. Some of them play important roles in enabling polypeptides to reach biologically active forms by serving as “detergents” and “proofreading apparatus” during protein synthesis and secretion^[11]. In pancreatic acinar cells, like pancreatic enzymes, Cpn60 follows the well-characterized secretory pathway, sequentially increasing along the RER-G-ZG secretory pathway, and finally being discharged into the pancreatic juice^[12-14]. In our former work, we have explored and analyzed the characteristic distribution of Cpn60 in the pancreatic acinar cells of rats, and an association of Cpn60 with lipase, amylase, and trypsinogen in the secretory pathway of normal rats^[1,5,6].

The interesting finding, in this study, was that lipase and chymotrypsinogen increased to almost two to three times their normal levels in the acinar cells of the diabetic rats. However, the increased enzymes were not aggregated and autoactivated within the acinar cells like what happened in AP. The acinar cells and the pancreatic tissues were

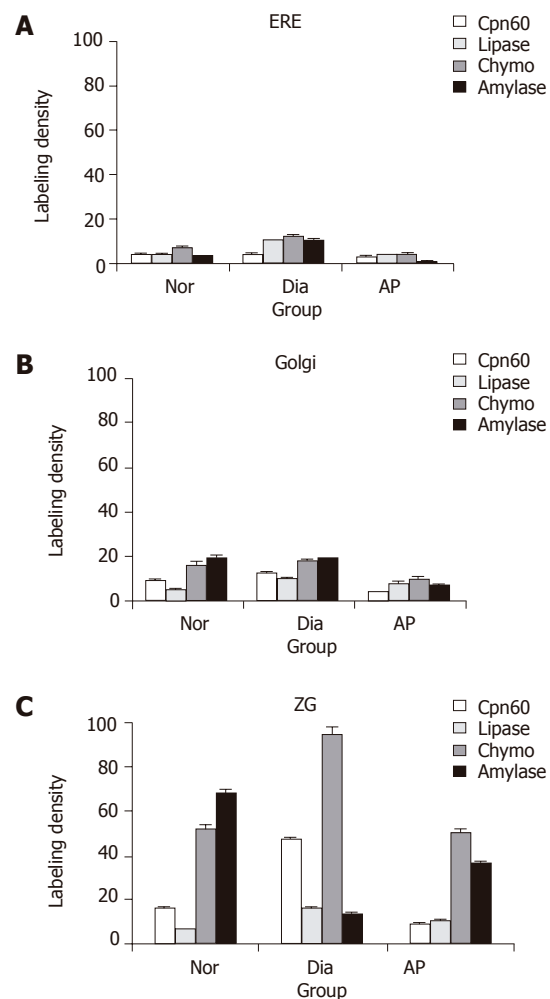


Figure 3 Immunolabeling densities of Cpn60 and the pancreatic enzymes in pancreatic acinar cells rats (particles/μm², mean ± SE). A: Rough endoplasmic reticulum; B: Golgi; and C: zymogen granules. Nor: normal group; Dia: diabetic group; Chymo: chymotrypsinogen.

not damaged in this situation. The important protective mechanism may be associated with enough chaperon capacity where Cpn60 content increased significantly (nearly threefold) in the pathological condition. In this regard, it has been reported that Cpn60 is involved in successful folding, sorting, and assembly of pancreatic oligomeric polypeptides, and prevents aggregation of the premature polypeptides^[15-17]. In addition, the increase of lipase and chymotrypsinogen and the decrease of amylase in diabetes may represent an adaptive process in which the diabetic body reduced starch digestion and then glucose absorption, and exhausted the fat and protein as the energy sources. In this regard, Nagy *et al.*^[18] found a temporarily increased lipase content, when plasma free fatty acid concentration was elevated. The present results demonstrated that the pancreatic lipase and chymotrypsinogen were affected by the body metabolism, and confirmed the complicated correlation between the hormones, enzymes, and the metabolic situation of glucose, fat, and proteins in the body.

AP is caused by different etiologic factors, and each of them affects firstly the pancreatic acinar cells, resulting

in the intracellular activation of trypsinogen, and then the lipolytic enzymes which, in turn, injure the acinar cells and the whole pancreas^[19,20]. The autodigestion of the pancreas by its own prematurely activated digestive proteases has been considered as an important event in the occurrence of AP^[21,22], but the mechanism is under elucidation. Recently, Lee *et al.*^[23] found that water immersion stress induced Hsp60 expression, prevented intrapancreatic trypsinogen activation, and then protected against cerulein-induced rat pancreatitis. Other reports also demonstrated that stresses, such as heat, acid, and osmotic shock, induced an increased transcription and production of Cpn60, and showed a protection for the pancreas, which was considered as an "adaptive cytoprotection"^[24-26]. It is well known that in the early stage of AP, progressive increases of amylase, trypsin, and lipase levels are found in the ascites and the plasma (hyperenzymemia), and there is also an increase of enzyme content in the individual acinar cells^[27,28].

In the present study, increased labeling densities of lipase and chymotrypsinogen were displayed in the AP sections. As an adaptive cytoprotection, the level of Cpn60 should increase; however, the data showed a decreased Cpn60 level and implied an insufficient chaperone capacity. This could in fact alter the steady-state equilibrium between some pancreatic secretory enzymes and chaperones, thus initiating premature enzyme aggregation and autoactivation in acinar cells. In this regard, Strowski *et al.*^[29] found that the pancreas reacted to various kinds of stresses with different inductions of Hsp mRNAs during cerulein-induced AP, and they hypothesized that failure to appropriately increase Hsp levels in response to high doses of cerulein might be a factor involved in the development of pancreatitis. The results of our present study also support this hypothesis.

In summary, the changes in Cpn60 levels coincide with the changes of pancreatic enzyme levels, mostly with lipase, chymotrypsinogen in the pancreatic acinar cells. The equilibrium between Cpn60 and the pancreatic enzymes in the acinar cells breaks in AP, and Cpn60 content decreases, thereby suggesting an insufficient chaperone capacity. This may promote pancreatic premature enzyme autoactivation and play a role in the AP pathogenesis. The findings suggest that replenishing Cpn60 and increasing chaperone capacity may be a new therapeutic clue of AP.

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• RAPID COMMUNICATION •

Determination of platelet-activating factor by reverse phase high-performance liquid chromatography and its application in viral hepatitis

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Abstract

AIM: To detect the platelet-activating factor (PAF) and the plasma or serum levels of tumor necrosis factor- α (TNF- α), malondialdehyde (MDA), endotoxin (ET) and to discuss their significance in various types of viral hepatitis.

METHODS: PAF, TNF- α , MDA, and ET levels in 60 controls, 16 cases of acute viral hepatitis, 71 cases of chronic viral hepatitis, 19 cases of severe viral hepatitis were detected by reverse phase high-performance liquid chromatography (rHPLC), bio-assay, ELISA, thiobarbituric acid (TBA), and limulus lysate test (LLT), respectively.

RESULTS: The rHPLC was more sensitive and specific than bio-assay ($r = 0.912$, $P < 0.01$). The plasma levels of PAF, TNF- α , MDA, and ET in patients with viral hepatitis were higher than those in controls ($P < 0.01$).

CONCLUSION: rHPLC is more reliable and accurate for the detection of PAF.

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Key words: Platelet-activating factor; Malondialdehyde; Endotoxin; rHPLC; Viral hepatitis

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INTRODUCTION

Platelet-activating factor (PAF) is an autocoid mediator which controls intra- and extra-cellular signal transduction^[1]. PAF is produced by a variety of cells including platelets, macrophages, neutrophils, and endothelial cells. Intrahepatic PAF is mainly secreted from Kupffer cells and hepatic sinusoidal endothelial cells. It activates platelets, leukocytes, and smooth muscle contraction. It was reported that PAF, as a mediator of lipid, plays an important role in the occurrence of hepatocyte injuries^[2]. Direct infusion of PAF into the liver can result in the rapid release of superoxide and direct damage to liver cells^[3,4]. PAF is also a mediator necessary for the development of endotoxin (ET) damage^[5]. In endotoxic shock, the production of PAF in tissues is significantly increased. When the concentration of PAF is extremely low in the body, PAF manifests a short half-life of only 30 s and is then transformed rapidly into lyso-PAF without biological activity. PAF is hard to link to the carriers of protein due to its low immunogenicity, which makes the explanation difficult in the antibody preparation. Consequently, the current biological and immunological techniques cannot meet the clinical requirements^[6]. Based on the biological determination, we used reverse phase high-performance liquid chromatography (rHPLC) to determine plasma PAF, tumor necrosis factor- α (TNF- α), malondialdehyde (MDA), and ET in patients with viral hepatitis and further clarified their effect and significance in all types of viral hepatitis in order to study the mechanism of viral hepatitis.

MATERIALS AND METHODS

Study population

Between January 2001 and June 2002, 106 patients of both sexes diagnosed as viral hepatitis at the Department of Infectious Diseases of our hospital were recruited in the study. The patients consisted of 16 cases of acute hepatitis, 26 cases of chronic light hepatitis, 20 cases of chronic moderate hepatitis, 25 cases of chronic severe hepatitis ($n = 25$) and 19 cases of severe hepatitis, ranging in age from 40 to 61 years, with a mean age of 49.5 ± 10.4 years. All the patients were diagnosed by the specialists of the Department of Infectious Diseases in accordance with the diagnostic criteria for viral hepatitis recommended on the 10th National Viral Hepatitis and Hepatopathy Conference in Xi'an 2000. All the patients did not receive

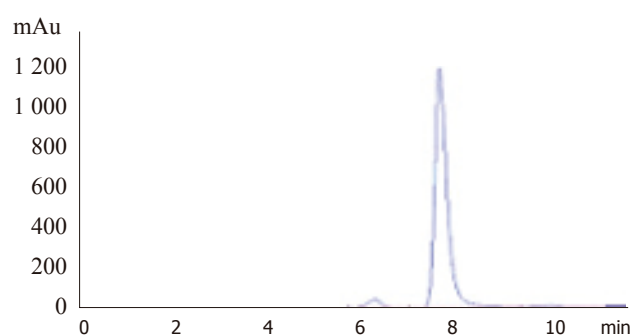


Figure 1 Standard C18 PAF. The horizontal and vertical axes represent the retention time (min) and milli-absorption unit (mAu) respectively, by which the peak area was calculated.

any immunomodulators and any drugs within 2 wk prior to this study.

Healthy control subjects ($n = 60$) without any thrombotic or communicable diseases were recruited from the blood center.

Blood PAF levels were measured in patients and normal volunteers. We also measured the plasma or serum levels of $\text{TNF-}\alpha$, ET, and MDA.

Agents and devices for PAF analysis by rHPLC

Agilent 1100 series HPLC, water chromatographic column (3.9 mm \times 150 mm), Lab-line® Aquawave™ ultrasonic cleaner, standard lyso-PAF-C16 and PAF-C18 (chromatographic grade) were from Sigma Company.

Assay of ET, MDA and $\text{TNF-}\alpha$

$\text{TNF-}\alpha$ was assayed with ELISA. The testing kits were from Jingmei Biological Engineering Co., Ltd.

ET was determined by limulus lysate test (LLT). The testing kits were from Shanghai Yi-Hua Clinical Medicine Technology Co., Ltd.

MDA was determined by thiobarbituric acid (TBA). The testing kits were from Nanjing Jian-Cheng Biological Engineering Research Institute.

PAF assay

Plasma was separated from whole blood by centrifugation at 3 000 r/min for 10 min and then stored at $-70\text{ }^{\circ}\text{C}$ until assay. PAF was assayed as previously described^[7]. The mobile phase was required according to chromatographic condition, the velocity of flow was 1.0 mL/min, the column temperature was at $25\text{ }^{\circ}\text{C}$ (Figure 1) and the determination wave was 208 nm. The PAF values of 20 randomly selected samples were determined by the biological technique established in our laboratory.

Statistical analysis

The descriptive values of variables were expressed as mean \pm SD. Categorical data were compared between the two groups with the Cochran's Q test. The correlations between laboratory results were evaluated by Pearson's correlation test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed by SPSS for Windows.

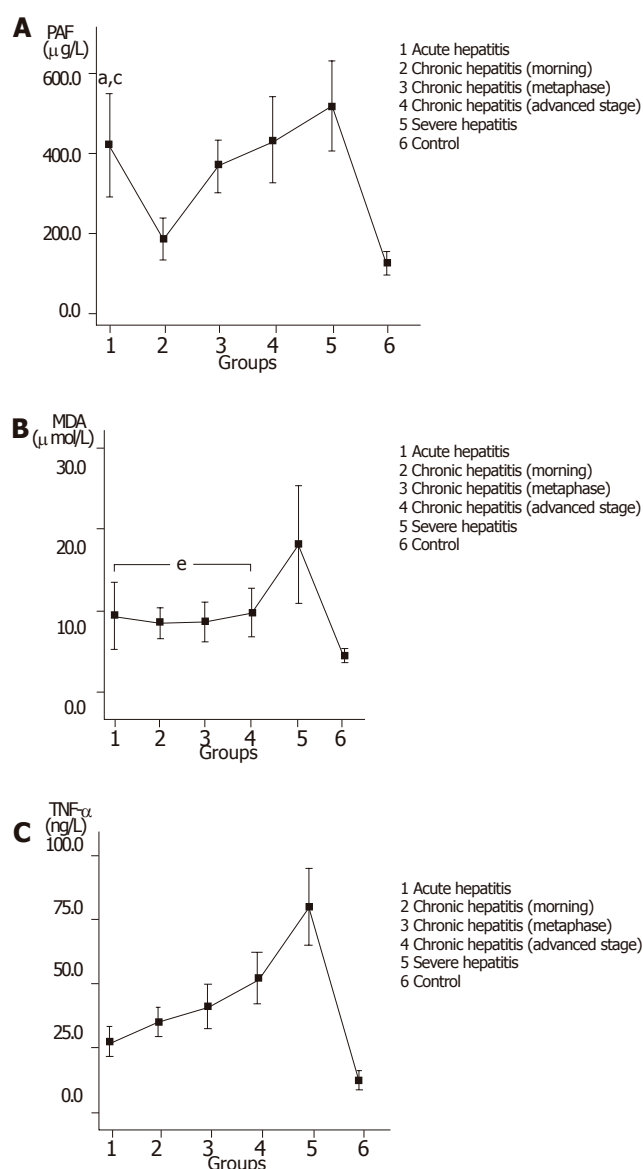


Figure 2 Plasma or serum levels of PAF (A), MDA (B) and $\text{TNF-}\alpha$ (C) in patients and controls. The values of PAF, $\text{TNF-}\alpha$ and MDA in patients with hepatitis were significantly higher than those in controls ($P < 0.01$). ^a $P < 0.05$ vs group 3, ^c $P > 0.05$ vs group 4, ^e $P > 0.05$ vs every two groups in groups 1-4.

RESULTS

In the assay of PAF by rHPLC, the retention times of lyso-PAF and C18 PAF were 6.2 and 7.8 min, respectively. The correlation analysis was performed for the results in the chromatography group and the biological technique group ($r = 0.912$, $P < 0.01$).

The ET value of over 50 ng/L represented the positive finding in endotoxemia detection^[6]. The detection rate of endotoxemia was 51.9%, 55.9%, 67.8%, and 0% in the patients with acute hepatitis, chronic hepatitis, severe hepatitis, and in the controls, respectively. The positive detection rate in the patients was significantly higher than that in the controls ($P < 0.01$).

The values of PAF, MDA and $\text{TNF-}\alpha$ in the patients and controls are shown in Figures 2A-C.

DISCUSSION

The biological assay or radio immunoassay of PAF fails to manifest satisfactory specificity and sensitivity, because PAF in the body is rapidly transformed into lyso-PAF without biological activity^[8]. In addition, the procedure is complicated and the results may be influenced by the individual difference of rabbit thrombocytes as well as the surgeon's skill in plate preparation and sample application^[9]. The immunoassay may also be limited by antibody preparation that is actually difficult to complete. A desirable correlation is indicated between rHPLC and biological assay. The assay of PAF by rHPLC can lead to the effective separation and then the assay of PAF components (including C16 and C18) with different structures in the assayed sample not related to the biological activity of PAF. Thus, the reference values obtained are notably higher than those obtained by biological assay, contributing to a great increase in accuracy.

The liver plays an important role in removing ET by Kupffer cells and macrophages^[10]. In our study, the detection rate of endotoxemia was 52.80%, 56.41%, and 69.57% in acute, chronic and severe hepatitis, respectively. Besides direct damage to the liver, ET is able to induce and release a large amount of inflammatory mediators including PAF and TNF- α by activating Kupffer cells, macrophages and hepatic sinusoidal endothelial cells. These mediators are not only involved in various biological effects of ET, but also exhibit their own biological effects by inducing generation of other inflammatory mediators, resulting in inflammation and tissue injuries in the body by various mechanisms^[11,12]. PAF may cause many symptoms of endotoxic shock, including hypotension, cardiac function inhibition, and blood plasma exudation. The specific receptor antagonist of PAF has preventive and therapeutic effects on endotoxic shock. ET also has certain synergistic effect on PAF. Animal experiments showed that simultaneous use of ET and PAF could lead to severe endotoxic shock, granulopenia, and bowel necrosis. The TNF- α gene expression induced by ET is also related to PAF. The PAF antagonist can be used to effectively reduce the level of TNF in blood plasma and partially inhibit intrahepatic synthesis of TNF mRNA, indicating that TNF mRNA may be synthesized in a PAF-dependent manner or in a PAF-independent manner^[13].

MDA is generated through lipid peroxidation. It can damage the complete membrane structure of hepatocytes and organelles, activate Kupffer cells, lead to endotoxemia and is closely correlated with the occurrence of hepatopathy^[14]. In this study, PAF in patients with acute hepatitis was notably higher than that in patients with early- or medium-phase chronic hepatitis, suggesting that ET, PAF, TNF, and MDA may result in the aggravation of liver injury^[15]. The PAF antagonist perfusion into the liver in advance can increase bile production in the affected liver, decrease the production of lipid peroxides (including MDA) and prevent energy damage by PAF. The value of

blood MDA is remarkably increased in the early stage of severe hepatitis and returns to the normal levels during convalescence (data not shown)^[16]. Therefore, MDA can be used as one of the indicators of diagnosis and prognosis of severe hepatitis.

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• RAPID COMMUNICATION •

Chloromycetin resistance of clinically isolated *E coli* is conversed by using EGS technique to repress the chloromycetin acetyl transferase

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Abstract

AIM: To explore the possibility of repression of chloromycetin (Cm) acyl transferase by using external guided sequence (EGS) in order to converse the clinical *E coli* isolates from Cm-resistant to Cm-sensitive.

METHODS: EGS directed against chloromycetin acetyl transferase gene (*cat*) was cloned to vector pEGFP-C1 which contains the kanamycin (Km) resistance gene. The recombinant plasmid pEGFP-C1+EGScat1+cat2 was constructed and the blank vector without EGS fragment was used as control plasmids. By using the CaCl₂ transformation method, the recombinant plasmids were introduced into the clinically isolated Cm resistant but Km sensitive *E coli* strains. Transformants were screened on LB agar plates containing Km. Extraction of plasmids and PCR were applied to identify the positive clones. The growth curve of EGS transformed bacteria cultured in broth with Cm resistance was determined by using spectrophotometer at A₆₀₀. Drug sensitivity was tested in solid culture containing Cm by using KB method.

RESULTS: Transformation studies were carried out on 16 clinically isolated Cm-resistant (250 µg/mL of Cm) *E coli* strains by using pEGFP-C1-EGScat1cat2 recombinant plasmid. Transformants were screened on LB-agar plates containing Km after the transformation using EGS. Of the 16 tested strains, 4 strains were transformed successfully. Transformants with EGS plasmid showed growth inhibition when grown in liquid broth culture

containing 200 µg/mL of Cm. In drug sensitivity test, these strains were sensitive to Cm on LB-agar plates containing 200 µg/mL of Cm. Extraction of plasmids and PCR amplification showed the existence of EGS plasmids in these four transformed strains. These results indicated that the Cat of the four clinical isolates had been suppressed and the four strains were converted to Cm sensitive ones.

CONCLUSION: The EGS directed against Cat is able to inhibit the expression of Cat, and hence convert Cm-resistant bacteria to Cm-sensitive ones. Thus, the EGS has the capability of converting the phenotype of clinical drug-resistant isolates strains to drug-sensitive ones.

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Key words: External guide sequence; Drug-resistant bacteria; Conversion of drug resistance

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INTRODUCTION

Drug resistance in pathogenic bacteria is a problem of major clinical importance, which has not been effectively solved yet. The traditional approach to this problem is searching for novel antibiotics. However, this is expensive and time consuming, and the bacteria can quickly develop drug resistance to these novel antibiotics. With advances in gene therapy, ribozyme technology^[1,2] appeared following antisense RNA technique^[3-6], which though is abstract of the activity of catalysis and cleavage. Then, external guide sequence (EGS) technique^[7] was found, with the advantages of both ribozyme and antisense RNA. EGS, a synthetic gene coding for small oligoribonucleotides, is able to form complexes with the mRNA encoded by target gene. The complexes are recognized as substrates of RNase P^[8-11]. Then, EGS directs RNase P to cleave and inactivate the target mRNA. Therefore, the EGS technique is a method to block a particular gene by inhibiting the translation of its mRNA^[12-14]. In 1997, by designing EGS directed against chloromycetin (Cm) acyl transferase (*cat*), Sidney Altman successfully applied EGS technique in engineered bacteria to convert the Cm-resistant bacteria into Cm-sensitive

ones, and hence paved a new way to solve the drug resistance problem^[15]. In this study, we investigated the feasibility of applying EGS technique in converting the phenotype of clinically isolated *E. coli* from Cm-resistant to Cm-sensitive.

MATERIALS AND METHODS

Materials

The *E. coli* samples were clinically isolated from Affiliated Tongji Hospital of Huazhong University of Science and Technology between 2001 and 2002, from which 16 strains resistant to Cm 250 µg/mL and sensitive to kanamycin (Km) 50 µg/mL were selected as test strains. Plasmid pEGFP-C1 and pKB EGS cat1+cat2 were kindly provided by Dr. Sidney Altman (Yale University, USA). The plasmid pKB EGS cat1+cat2 was constructed according to the method in the literature^[15].

Methods

Construction of pEGFP-C1-EGS cat1+cat2 recombinant plasmids A 510-bp EGS fragment directed against Cm acetyl transferase gene (*cat*) was cloned into pEGFP-C1 plasmid containing kanamycin-resistant gene between EcoRI and I sites. The EGS includes: M1RNA promoter, EGScat1, hammerhead sequence (HH1, HH2), EGScat2, HH3, and M1 terminator. The recombinant pEGFP-C1 plasmid containing EGScat1+cat2 was named K₃ plasmid, while pEGFP-C1 plasmid that had Km resistance gene but without EGS fragment was named as K plasmid.

Identification of partial phenotypes of clinically isolated *E. coli*: (1) *Purification of the test bacteria* The clinically isolated bacterial strains were streaked on the LB plates and cultured for 6-8 h. A single colony on the plates was selected for Gram staining and its cellular configuration was checked under the microscope. Other single colony was then taken and diluted to 10⁻⁸. The clone grown on plates with 30-300 colonies was taken for tests of Cm drug resistance and Km drug sensitivity again, and then Gram stained for morphology check. These steps were repeated until pure bacterium strains were obtained^[16]; (2) *Extraction of plasmids of the test bacteria and determination of plasmid incompatibility* The methods for plasmid extraction and incompatibility determination were described by Huang^[17]; (3) *Determination of spontaneous mutation rate of the test bacteria* The spontaneous mutation rate of testing bacteria to Km was determined for colonies grown on LB and LB+Km plates by using dilution plate counting method^[18]; (4) *Determination of logarithmic growth stage of test bacteria* Bacterial growth curve were plotted based on bacterial optical density (*A*₆₀₀)^[19].

Transformation of test bacteria by pEGFP-C1-EGScat1+cat2 recombinant plasmids and identification:

(1) *Transforming test* The competent cells of testing bacteria were prepared by using CaCl₂ method, and were transformed respectively with plasmids K₃ and K^[20]. The transformants that received K₃ plasmid were designated as testing bacteria, and those transformed with K plasmid without EGS were used as plasmid

controls. The competent cells from original testing bacteria were mock-transformed (without plasmid) and served as strain controls. Competent cells were inoculated on LB plates containing 50 µg/mL of Km after transformation, followed by incubation at 37 °C for 12-16 h; (2) *Extraction of plasmids and total DNA* Plasmid was extracted by using alkaline cleavage method^[17] and total DNA was extracted by using the boiling method^[21]. Positive clones were selected and transferred into Eppendorf tubes containing 20 µL sterilized water and boiled for 10 min. The supernatant was collected after centrifugation; (3) *PCR amplification* The primers were designed specifically for the inserted EGS on the vector. The amplified fragment containing EGScat1 and cat2 was 391 bp. The primer pairs used for PCR amplification were 5'-AGCCTGACCGATGATGTTG-3' and 5'-TCCTCACGGACTCATC AGAC-3'. The PCR reactions were performed at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1.5 min, total 30 cycles. The PCR products were electrophoresed on a 1.5% agarose gel; (4) *Cm sensitivity test of the transformants* After transformation, the colonies grown on plates and the original colonies were re-inoculated on the LB plates containing 200 µg/mL of Cm and plates containing 50 µg/mL of Km, and incubated at 37 °C for 8-12 h. Then, their growth conditions on these two plates were checked and recorded; (5) *Growth determination of transformants in liquid broth* The colonies (K₃, K plasmid transformants and original bacteria) were separately inoculated into 200 mL of broth culture containing Cm (200 µg/mL) with the inoculating concentration *A*₆₀₀ = 0.05, and incubated in an orbital shaker at 37 °C and 200 r/min. A 0.5 mL bacterial suspension was sampled hourly and its *A* value was determined. Growth curve was plotted according to the constantly collected *A* values. The transformants were also incubated at the same concentrations into PA bottles at 37 °C and the bacterial growth was observed hourly as well.

RESULTS

Study of the partial phenotypes of the clinically isolated *E. coli* strains' rates of spontaneous mutation

Sixty-one Cm-resistant but Km-sensitive *E. coli* strains were clinically isolated. Of them, 16 strains were selected as test strains for their comparatively low simultaneous mutation rate. However, the test strains sensitive to Km were still suitable to present the spontaneous mutation and hence obtain Km resistance after culturing in a big scale. Among them, strains 16, 20, 3 900, and 6 470 showed the lowest mutation rate of Km drug resistance, being 8.85×10⁻⁶, 3.36×10⁻⁶, 6.57×10⁻⁷, and 2.96×10⁻⁷, respectively. The data of others were little higher than 10⁻⁵.

Plasmids' profile of the test bacteria The plasmid profile of the test strains showed that each contained 2-3 plasmid bands. Introducing the plasmids extracted from test bacteria into *E. coli* DH5α (no indigenous plasmids) indicated that Cm-resistant gene was located in the plasmids. The existence of resistance plasmids in the test strains may account for the difficulty of introducing EGS

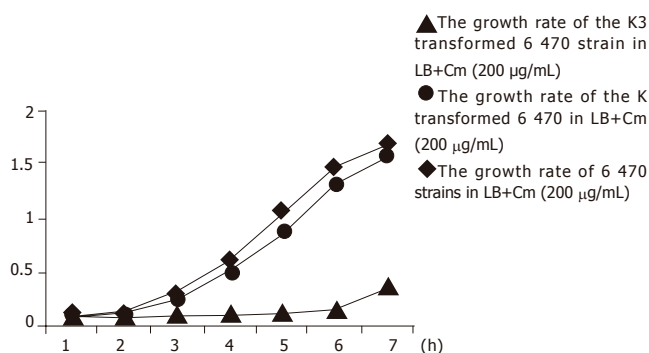


Figure 1 Growth curve of the transformants in the broth containing Cm after K3 plasmid and K plasmid were introduced into 6 470 strains of Cm-resistant *E. coli*.

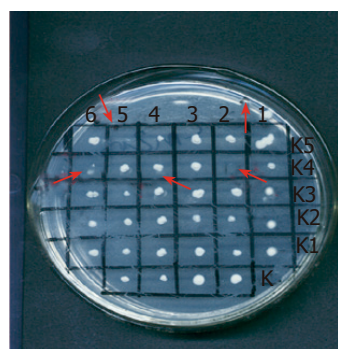


Figure 2 Susceptibility test of the transformed bacteria in solid culture containing Cm (200 µg/mL) after K3 plasmids and K plasmids were introduced into 6 470

plasmid into the cells. Furthermore, the DH5α derivatives transformed with the plasmids extracted from test bacteria were hard to be transformed again with K3 and K. This suggested the plasmid incompatibility between original plasmids and K or K3 plasmids.

Determination of logarithmic phase The bacterial growth curve revealed that the logarithmic growth stage was from 4 to 8 h after being inoculated in the broth. In this study, the fresh culture of test strains was harvested in this period to prepare competitive cells.

Growth rate of the transformed bacteria in liquid broth culture

Four strains, 16, 20, 3 900, and 6 470 were transformed with K3 successfully. Kinetic growth study demonstrated that these four strains transformed with K3 plasmid exhibited growth inhibition in the broth containing 200 µg/mL of Cm (Figure 1). The growth inhibition became apparent at 2 h and peaked at 3 and 4 h, but active growth resumed at 5 h. In contrast, neither bacteria transformed with K plasmid nor original bacteria with mock-transformation showed any growth inhibition in the incubation condition described above. Their concentrations increased steadily as demonstrated by A_{600} values. Although the number of bacteria transformed with EGS began to elevate at 5 h, the degree of growth was much lower than that of the original bacteria. In PA bottles, the K3 plasmid transformed bacterial culture was

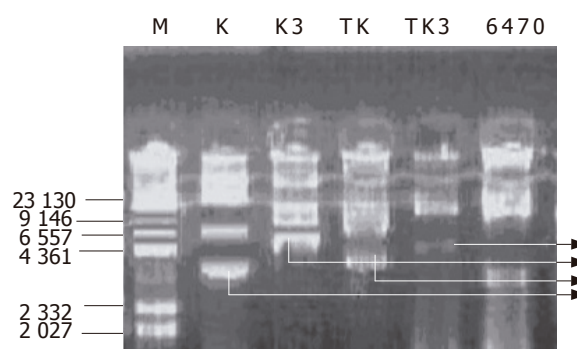


Figure 3 Results of rapid plasmid extraction after K3 plasmid and K plasmid were introduced into 6 470, K and TK, K3 and TK3 can be detected in similar bands.

still clear at 6 h to the naked eye and started to be turbid at 8 h, while the culture for K plasmid transformed bacteria or mock-transformed original bacteria started to be turbid at 3 h and was highly turbid at 6 h. The above findings demonstrated that EGS gene was able to convert bacteria from Cm-resistant to Cm-sensitive phenotype. All of the four strains that obtained their phenotypic conversion of drug resistance showed a lower spontaneous mutation rate, which may contribute to their easy transformation because of their stability. They exhibited very few variant strains when cultured on Km plates, and thus the transformants could be easily identified and collected.

Drug sensitivity test of transformed bacteria

Drug sensitivity test showed that the four strains transformed with K3 plasmid not only obtained sensitivity to Cm in liquid broth culture but also became sensitive to Cm in LB+Cm (200 µg/mL) solid culture (as indicated in Figure 2). The bacteria transformed with K plasmid and the original isolates grew well in LB+Cm (200 µg/mL) solid culture.

Determination of plasmid extraction from testing bacteria transformed by pEGFP-C1-EGS cat1+cat2 and identification of clones by PCR

Determination of the plasmids in transformed bacteria

Four strains of transformed bacteria were subjected to the determination of the plasmid extraction. Figure 3 is the map of rapid extraction of plasmid and Figure 4 is the map of plasmid extraction after the transformants were cultured for 2 d. Figure 3 shows that the K and K3 can be detected in the strains transformed with K and K3, respectively. This indicated that K3 and K plasmids had been introduced into the cells successfully. In Figure 4, TK had the same bands as K, while TK3 had no similar bands as K3, indicating that the K3 plasmid was lost or had been degraded after 2 d of culturing. This may explain why the transformant retained its growing capacity gradually after several hours of culturing in the broth medium.

Identification of EGS gene by PCR The colonies transformed with K3, which did not grow on LB+Cm (200 µg/mL) plate (restoration of Cm sensitivity) but grew well in the corresponding LB+Km (50 µg/mL) plate, were

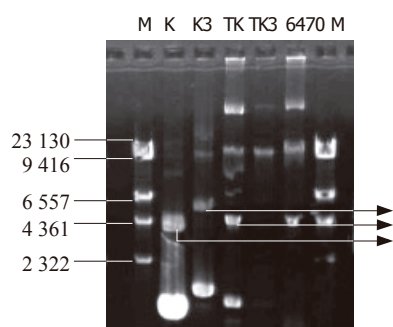


Figure 4 The map of plasmid extraction after the transformants were cultured for 2 d, K and TK can be detected in similar bands, while TK3 had no similar bands as K3, indicating the K3 plasmid was lost or had been degraded. M: Marker; lane K: control plasmid; K3: EGS plasmid; TK, 6 470 transformed with K plasmid; TK3, 6 470 transformed with K3; 6 470, original Cm-resistant 6 470.

used for PCR amplification of EGS fragments. K3 plasmid was used as positive control and clones transformed with K plasmid, original testing bacteria and DNA-free distilled water were used as negative controls. As shown in Figure 5, lanes 5, 6, and 7 were clones transformed with K3 plasmid that did not grow on LB+Cm plate, showing that these clones contained the EGS gene fragment (391 bp, lane 8). Lanes 4 and 9 were original test bacteria and K plasmid transformed bacteria, showing no EGS gene fragment in these strains. Lane 3 was the clone that initially resumed Cm sensitivity but lost the sensitivity 2 d later on LB+Cm plate. PCR amplification failed to detect any EGS band, suggesting the loss of EGS plasmid in this clone.

DISCUSSION

The problem of microbial drug resistance is growing severely year after year clinically. To cope with this problem, destroying the drug resistance gene is an effective and potential approach, in which no effective method has so far been achieved^[22-24]. In 1997, Sidney Altman introduced EGS technique, which converted the Cm resistance successfully. On the basis of the study on engineered bacteria by Altman, this study further introduced the EGS gene into the clinically isolated bacterial strains. Our study demonstrated EGS-mediated conversion of a Cm-resistant to a Cm-sensitive phenotype, and further showed the successful conversion of drug resistance by the introduction of EGS gene not only in laboratory bacteria but also in clinically isolated bacteria. This indicates a potential clinical utilization.

Mutation is a common phenomenon present in microorganisms. The clinically isolated bacterial strains are characterized with even higher spontaneous mutation rate than lab strains. In the early period of our experiments, it was found that the competent cells of the control bacteria sensitive to Km not transformed with plasmids and originally sensitive to Km still grew on the Km plates. At first, it was mistakenly rendered as the result of impurity of test strain. After further purification and spontaneous mutation study, we understood that it has resulted from

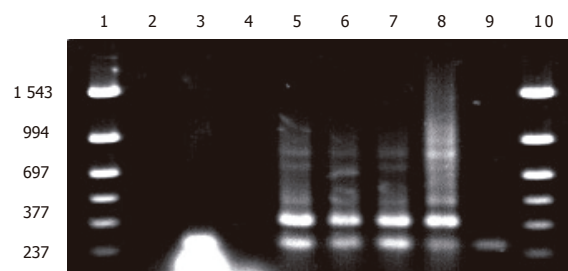


Figure 5 Electrophoresis of PCR amplification of EGS genes in the transformed bacteria after K plasmid and K3 plasmid introduced into 6 470. Lanes 1 and 10 Mmarker; Lane 2, blank control; lane 3, transformants cultured 2 d more; lane 4, original 6 470 strains; lanes 5, 6 and 7: 6 470 derivatives transformed with K3; lane 8: plasmid K3 (positive control); lane 9: K plasmid transformants (negative control).

high mutation rate of the clinically isolated bacteria. Mutated test strains obtained Km resistance, lost Km-sensitiveness, and therefore made selecting transformants difficult. To solve this problem, our efforts focused on selecting stable strains with low spontaneous mutation rate. At the end of the procedure, 16 comparatively stable strains were screened from the clinically isolated bacterial strains.

In this study, only 4 of 16 test strains were converted from drug-resistant to drug-sensitive phenotype. The low efficiency of conversion may be ascribed to the low transformation efficiency. Firstly, as *Enterobacteriaceae*, test strains were hard to be transformed for their native low transformation frequency^[25]. Secondly, the low transformation rate can be contributed to the existence of host plasmids in test strain which may result in plasmid incompatibility. Thirdly, although the criteria for screening positive transformants in the culture medium containing Km were set up, not all of the transformed bacteria were desired transformants due to the spontaneous mutation of the testing bacteria. We believe that even if we select other sensitive antibiotics to testing bacterial strains, spontaneous mutation would also happen. So suitable methods to screen positive transformants were abstract. Km resistance in this study did not function as an effective selection marker due to the spontaneous mutation of the test strains. To increase the efficiency of conversion, we tried to apply one-step method^[26] and electroporation transformation method, but the results were also not satisfactory. Although we can construct recombinant plasmids and insert a fluorescence enzyme's gene into the vector (EGFP site at pEGFP-C1 vector) to aid in screening, it may increase the transforming difficulty or render the transformed bacteria. In order to reduce the false positive clone in transformation assay, the original bacteria with high spontaneous mutation rate were excluded. At the same time, we found that the efficiency of transformation could be enhanced by using SOC medium and by collecting cell culture at early logarithmic phase. Transformation could be facilitated by washing cell culture with CaCl₂ solution for 2-3 times instead of once as well. This might be contributed to the increased permeability of the *E. coli* wall.

PCR amplification was used to identify positive clones. In contrast to the spontaneously mutated bacterial strains, the transformants obtained through transformation grew slowly on the Km plates. On the other hand, long time culturing resulted in the loss of plasmids. Thus, it was difficult to identify the EGS transformants by plasmid extraction. In this study, PCR was applied to identify the presence of EGS in the EGS transformed bacteria and it worked effectively. Certainly, further study is to perform Northern blot to know the attenuation of mRNA transcribed from cat gene, which could further definitely verify the roles of EGS. As our conditions are limited, the experiment was not done.

EGS as a sort of synthesized oligonucleotides can combine the target RNA to form the substrate similar to pre-tRNA, which can be cleaved by RNase P and lose the ability of further gene expression. The targeted mRNA is cleaved but EGS itself is not cleaved^[7]. EGS can function continuously. In this study, however, we found that the positive clones regained the Cm resistance after being cultured in broth containing Cm for 8 h and then on solid culture for 2 d. No EGS was detected and no plasmid could be extracted in clones that regained Cm resistance. This demonstrated that EGS plasmid had been lost in the host because of continuous culture. The reason might be due to an incompatibility between indigenous and exogenous plasmids.

Our experiments demonstrated that culturing on Km plates facilitated EGS plasmid inheritance, while culturing on the Cm plates was prone to result in the loss of EGS plasmid. This study also suggested the need for more effective methods to introduce EGS into the bacterial cells and to maintain its stability. Though a lot of challenges were involved in this study, transformants were still obtained. The results reflected the effectiveness of the EGS transforming drug-resistant bacteria, and provided a foundation for the clinical application of EGS.

In recent years, although RNA interference technique has been developed, no RNA interference phenomenon was observed in prokaryotes^[27-30]. Thus, the EGS technique still is the most prospective way to solve bacterial drug resistance. As to the development of EGS technique, Sidney Altman put forward that enhancing the promoter, increasing the copy of EGS, augmenting the binding sites of EGS and stabilizing the EGS-mRNA complex are the potential methods. Furthermore, combined with nanotechnology, the EGS technology could be more promising. Conclusively, to improve this study, more measures should be explored for further investigation.

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• RAPID COMMUNICATION •

Peritoneal lavage cytology and carcinoembryonic antigen determination in predicting peritoneal metastasis and prognosis of gastric cancer

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Abstract

AIM: To evaluate the role of peritoneal lavage cytology (PLC) and carcinoembryonic antigen (CEA) determination of peritoneal washes (pCEA) in predicting the peritoneal metastasis and prognosis after curative resection of gastric cancer.

METHODS: PLC and radioimmunoassay of CEA were performed in peritoneal washes from 64 patients with gastric cancer and 8 patients with benign diseases.

RESULTS: The positive rate of pCEA (40.6%) was significantly higher than that of PLC (23.4%) ($P < 0.05$). The positive rates of PLC and pCEA correlated with the depth of tumor invasion and lymph node metastasis ($P < 0.05$). pCEA was found to have a higher sensitivity and a lower false-positive rate in predicting peritoneal metastasis after curative resection of gastric cancer as compared to PLC. The 1-, 3-, and 5-year survival rates of patients with positive cytologic findings or positive pCEA results were significantly lower than those of patients with negative cytologic findings or negative pCEA results ($P < 0.05$). Multivariate analysis indicated that pCEA was an independent prognostic factor for the survival of patients with gastric cancer.

CONCLUSION: Intraoperative pCEA is a more sensitive and reliable predictor of peritoneal metastasis as well as prognosis in patients with gastric cancer as compared to PLC method.

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Key words: Stomach neoplasm; CEA protein; Peritoneal metastasis; Prognosis

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INTRODUCTION

Peritoneal metastasis is the most common mode of relapse of gastric cancer after surgery and is the most frequent cause of death in patients with gastric cancer^[1-3]. Peritoneal recurrence develops from micrometastasis originating from peritoneal free cancer cells. Therefore, it is very important to examine the presence or absence of free cancer cells in the peritoneal cavity at the time of surgery^[4,5]. PLC is the gold standard for assessing the presence of peritoneal dissemination of gastric cancer, but its sensitivity is relatively low, ranging 14-21% in gastric cancer involving the serosa^[6-8]. Recently, several new methods for detecting micrometastasis, including immunohistochemical and biological methods have been developed^[9-11]. However, these diagnostic techniques are time-consuming and laborious compared to conventional cytological method. Therefore, a new and more sensitive method for the early detection of peritoneal metastasis is required.

In this paper, we have reported the clinical significance of a new method to detect peritoneal micrometastasis in combination with cytological method and measurement of pCEA level in peritoneal washes.

MATERIALS AND METHODS

Patients

Between December 1995 and December 1997, 64 patients with histologically confirmed gastric cancer underwent surgery at the Department of General Surgery of First Affiliated People's Hospital of Shanghai Jiaotong University. All patients underwent either a total or a partial gastrectomy with lymph node dissection and received no preoperative chemotherapy or radiotherapy before the surgery. These 64 cases included 42 male and 22 female patients with an average age of 59 (range 34-84 years) years. All specimens were histologically examined by HE staining according to the general rules of the Japanese Classification of Gastric Carcinoma^[12]. Seven patients with

the maximum depth of tumor invasion at mucosal or submucosal level, were diagnosed as early gastric cancer. The other 57 cases were diagnosed as advanced gastric cancer with invasion deep into the gastric wall. In this study, eight patients with benign diseases such as peptic ulcer or cholecystolithiasis served as controls. The follow-up period ranged 9-74 mo and the median follow-up period was 39 mo.

Examination of peritoneal washes

The study consisted of 64 patients with gastric cancer and 8 patients with benign disease. After laparotomy, 50 mL physiological saline was introduced into the right upper quadrant or the Douglas pouch immediately and then 20 mL fluid was collected. The fluid sample was immediately centrifuged for 5 min at 1 500 r/min. The sediment of each fluid sample was smeared on a glass slide. The slide was fixed in 99% alcohol, stained with HE and examined for the presence of cancer cells by an experienced pathologist. The supernatant of each sample was concentrated by ultrafiltration. CEA levels of the concentrated supernatant (pCEA) were measured with a radiometric immunoassay kit (Delfia CEA kit, Wallac Oy, Turku, Finland) and expressed as ng/g of protein. The cut-off level was set according to Takayuki method (pCEA level ≥ 100 ng/g of protein was defined as positive)^[13].

Statistical analysis

All the statistical analyses were done with SPSS statistical software. The χ^2 test was used to compare the positive results in CEA level and cytological examination. The survival rate was calculated by the Kaplan-Meier method and statistical difference was evaluated by the long-rank test. A Cox proportional hazard model was established to identify the independent prognostic factors.

RESULTS

Correlation between PLC or pCEA and clinicopathologic parameters

All the eight patients with benign diseases had a negative PLC finding or a pCEA level below the positive standard. Fifteen of sixty-four patients with gastric cancer had a positive PLC finding and an elevated pCEA level. Among the 49 patients with negative PLC findings, 11 had an elevated pCEA level. The overall pCEA positive rate in 64 patients with gastric cancer was 40.6% (26/64), which was significantly higher than that of PLC [23.4% (15/64), $P < 0.05$]. We analyzed the correlation between PLC or pCEA positive rate and various clinicopathologic factors. The PLC and pCEA positive rates were significantly associated with the depth of tumor invasion and lymph

node involvement. The patients with serosal invasion had a significantly higher PLC or pCEA positive rate than those without serosal invasion ($P < 0.01$). The pCEA positive rate in patients with lymph node involvement was significantly higher than that in patients without lymph node involvement ($P < 0.05$). Though the PLC positive rate in patients with lymph node involvement was also higher than that in patients without lymph node involvement, the difference did not reach statistical significance ($P > 0.05$, Table 1).

Predicting values of PLC and pCEA for postoperative peritoneal recurrence of gastric cancer

Thirty-seven of sixty-four patients with gastric cancer had various postoperative recurrences (57.8%). Among the 37 patients with recurrence, 19 had peritoneal recurrence within 2 years after the surgery. The peritoneal recurrence rate was 29.7%. The accuracy, sensitivity, specificity, positive and negative predictive values, false negative and positive rates of PLC or pCEA in predicting peritoneal recurrence are shown in Table 2.

Correlation between PLC or pCEA and prognosis

The 1-, 3-, and 5-year survival rates were 53.3%, 13.3%, and 0.0%, respectively in patients with positive PLC findings, which were significantly lower than 87.8%, 71.4%, and 55.1% in those with negative PLC findings ($P < 0.05$). Similarly, a significant difference in survival rates between pCEA-positive group and pCEA-negative group was observed. The 1-, 3-, and 5-year survival rates were 46.2%, 23.1%, and 15.4% in pCEA positive group and 89.5%, 73.7%, and 60.5% in pCEA negative group ($P < 0.05$). Multivariate Cox survival analysis showed that PLC was not an independent prognostic factor, but pCEA was an independent prognostic factor with a relative risk rate of 9.046 ($P = 0.0092$).

Table 1 Correlation between the results of peritoneal washes and clinicopathological factors

Clinicopathological factors	N	PLC(+)		P	pCEA(+)		P
		n	%		n	%	
Tumor size							
≤4 cm	28	4	14.3	>0.05	9	32.1	>0.05
>4 cm	36	11	30.6		17	47.2	
Histological type							
Differentiated	30	5	16.7	>0.05	10	33.3	>0.05
Undifferentiated	34	10	29.4		16	47.1	
Serosal invasion							
Negative	27	2	7.4	<0.01	5	18.5	<0.01
Positive	37	13	35.1		21	56.8	
Lymph node involvement							
Negative	16	1	6.3	>0.05	3	18.8	<0.05
Positive	48	14	29.2		23	47.9	

Table 2 Results of PLC and pCEA in predicting peritoneal recurrence of gastric cancer after surgery (%)

	Accuracy	Sensitivity	Specificity	Positive predictive value	Negative predictive value	False negative rate	False positive rate
PLC	90.6	73.7	97.8	93.3	89.8	26.3	2.2
pCEA	85.9	94.7	82.2	69.2	97.4	5.3	17.8

DISCUSSION

Though radical surgery is routinely practised, tumor recurrence is frequent in patients with gastric cancer. Peritoneal recurrence of gastric cancer is one of the most common patterns of recurrence and predicts a very poor prognosis for patients with gastric cancer^[2,3,14,15]. Peritoneal dissemination was also prevalent in our current prospective study, which was observed in 19 of 64 patients (29.7%) following a potentially curative surgery. Peritoneal recurrence develops from peritoneal free cancer cells originating from primary lesion or metastatic lymph nodes^[6,14,15]. However, it is difficult to identify these free cancer cells in the peritoneal cavity before or during the surgery^[16]. In order to prevent postoperative peritoneal recurrence and increase the survival rate, it is important to find a more sensitive, accurate, and convenient method to detect the presence of free cancer cells in the peritoneal cavity and to eliminate these cells with effective measures such as adjuvant intra-peritoneal chemotherapy^[17,18].

PLC is the gold standard for assessing the presence of free gastric cancer cells in the peritoneal cavity^[6-8,19]. Cytology-positive patients are classified as stage IV of Union International Contrele Cancer (UICC) gastric cancer classification and curative surgery is impossible in such cases. The present study revealed that the PLC positive rate in patients with serosal invasion (35.1%) was significantly higher than that in patients without serosal invasion (7.4%), indicating that the PLC positive rate increases with the invasion of gastric serosa and the chance of peritoneal dissemination increases in case, the gastric serosa is infiltrated. However, 14 of 19 patients with postoperative peritoneal recurrence had a positive PLC finding and the other five cases were PLC negative. PLC showed an accuracy of 90.6%, a sensitivity of 73.7%, and a specificity of 97.8% in predicting peritoneal recurrence of gastric cancer. The positive and negative predicting value, the false negative and positive rates of PLC were 93.3%, 89.8%, 26.3%, and 2.2%, respectively, suggesting that PLC is a very useful method for predicting peritoneal recurrence of gastric cancer with a high accuracy and a high specificity. However, PLC lacks sensitivity and has a relatively high false negative rate. Some patients with negative PLC results may have recurrence in the form of peritoneal dissemination.

CEA is generally accepted as a specific marker of gastrointestinal tumor^[20,21]. Recently, measurement of CEA level and CEA RT-PCR assay in peritoneal washes have been used to detect the existence of free cancer cells in the peritoneal cavity^[10,22,23]. It was reported that RT-PCR based assay has a relatively high sensitivity but is time-consuming, relatively laborious and less practical^[10,22]. In the present study, pCEA levels in peritoneal washes were measured in order to detect the presence of free cancer cells in 64 patients with gastric cancer. The results showed that all the 15 patients with PLC positive findings were pCEA positive. However, 11 of 49 patients with PLC negative findings were also pCEA positive. The total pCEA positive rate was 40.6%, which was significantly

correlated with the presence of serosal invasion and lymph node metastasis. Thus, as a useful method for detecting the presence of free cancer cells in the peritoneal cavity and for clinical staging of gastric cancer, pCEA level determination is considered to be superior to PLC. In our study, 18 of 19 patients with postoperative peritoneal recurrence of gastric cancer were pCEA positive and only one patient who died of postoperative peritoneal recurrence was pCEA negative, suggesting that pCEA assay is a highly sensitive method to predict postoperative peritoneal recurrence with a relatively high accuracy and specificity as compared to conventional PLC method.

In addition, our results showed that the positive rates of PLC and pCEA assay were significantly correlated with survival. Patients with negative results in PLC or pCEA assay survived significantly longer than those with positive findings in PLC or pCEA assay. In a multivariate analysis, pCEA level was found to be an independent prognostic factor even when all the clinicopathological variables were considered.

In conclusion, pCEA is a potential predictor of peritoneal recurrence as well as poor prognosis in patients with gastric cancer. Intra-operative pCEA assay can be considered as a reliable method for patients with gastric cancer.

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• RAPID COMMUNICATION •

Small intestinal submucosa improves islet survival and function during *in vitro* culture

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Abstract

AIM: To evaluate the recovery and function of isolated rat pancreatic islets during *in vitro* culture with small intestinal submucosa (SIS).

METHODS: Pancreatic islets were isolated from Wistar rats by standard surgical procurement followed by intraductal collagenase distension, mechanical dissociation and Euroficol purification. Purified islets were cultured in plates coated with multilayer SIS (SIS-treated group) or without multilayer SIS (standard cultured group) for 7 and 14 d in standard islet culture media of RPMI 1640. After isolation and culture, islets from both experimental groups were stained with dithizone and counted. Recovery of islets was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation. Viability of islets after the culture was assessed by the glucose challenge test with low (2.7 mmol/L) and high glucose (16.7 mmol/L) solution supplemented with 50 mmol/L 3-isobutyl-1-methylxanthine (IBMX) solution. Apoptosis of islet cells after the culture was measured by relative quantification of histone-complexed DNA fragments using ELISA.

RESULTS: After 7 or 14 d of *in vitro* tissue culture, the recovery of islets in SIS-treated group was significantly higher than that cultured in plates without SIS coating. The recovery of islets in SIS-treated group was about twice more than that of in the control group. In SIS-treated group, there was no significant difference in the recovery of islets between short- and long-term periods of culture ($95.8 \pm 1.0\%$ vs $90.8 \pm 1.5\%$, $P > 0.05$). When incubated with high glucose (16.7 mmol/L) solution, insulin secretion in SIS-treated group showed a higher increase than that in control group after 14 d of culture (20.7 ± 1.1 mU/L vs 11.8 ± 1.1 mU/L, $P < 0.05$). When islets

were placed in high glucose solution containing IBMX, stimulated insulin secretion was higher in SIS-treated group than in control group. Calculated stimulation index of SIS-treated group was about 23 times of control group. In addition, the stimulation index of SIS-treated group remained constant regardless of short- and long-term periods of culture (9.5 ± 0.2 vs 10.2 ± 1.2 , $P > 0.05$). Much less apoptosis of islet cells occurred in SIS-treated group than in control group after the culture.

CONCLUSION: Co-culture of isolated rat islets with native sheet-like SIS might build an extracellular matrix for islets and provide possible biotrophic and growth factors that promote the recovery and subsequent function of islets.

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Key words: Islet culture; Islet survival; Islet function; Small intestinal submucosa

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INTRODUCTION

In the last 10 years, both the prevalence and incidence of diabetes have increased sharply all over the world and this disease has already become one of the global health care problems. For example, there are 14 000 000 patients suffering from diabetes in USA and 1 000 000 of them suffer from type I diabetes mellitus^[1]. Islet transplantation can control blood sugar effectively, reduce complications of diabetes and prevent hypoglycemia and insulin resistance caused by ectogenic insulin. Therefore, pancreatic islet cell transplantation may be an effective means for treating type I diabetes mellitus.

Human islet transplantation has been demonstrated to be a viable option for the treatment of type I diabetes mellitus^[2]. However, there are still many difficulties hindering the transplantation alleviating complications of the disease. A major limitation for its use as a standard treatment is the lack of available viable islets for transplantation.

To maintain islets of Langerhans in tissue culture provides a chance for islet storage after being isolated from pan-

creas before clinical transplantation. The storage period can be utilized to assess the function of the islets and to confirm microbiologic sterility of the preparation. In addition, it can increase purity of the islet preparation and to reduce the immunogenicity of islets^[3]. However, islet culture loses tissue mass over culturing time, which is one of its disadvantages^[4]. Given the importance of transplanting sufficient islets to increase the chance of successful graft^[5], any loss of islet tissue mass jeopardizes this possibility.

Both morphology and metabolic activities of cultured cells are affected by the composition of substrates on which they grow. Cultured cells may proliferate and/or perform their *in vivo* functions when cultured on substrates that closely mimic their natural environments. In fact, many commercially available matrices such as human extracellular matrix (ECM) derived from human placentas and matrigel (a soluble basement membrane extract from the Engelbreth-Holm-Swarm tumor) can be used in cell culture, which supports cell growth. Previous studies have showed that human islets cultured with bovine corneal endothelial cell matrix increase their ability to secrete insulin^[6].

SIS is a relatively acellular collagen-based matrix derived from porcine small intestine by mechanical removal of the mucosal and smooth muscle layers^[7]. The resulting cell-free translucent sheets are about 100 μ m thick. The collagen-based matrix comprised highly conserved collagen, glycoprotein, proteoglycan, and glycoaminoglycan in their native configuration and concentration. In addition, SIS includes various growth factors such as fibroblast growth factor-2 (FGF-2), TGF- β and vascular endothelial cell growth factor (VEGF), which promote cell growth^[8].

In 1966, Matsumoto *et al.*^[9] reported that inverted small intestine could replace large veins in dogs. Improved processing of this biomaterial (SIS) has made it readily available for tissue engineering studies. Extensive *in situ* tissue remodeling from SIS has been shown in both rat and canine models. SIS has been used as a scaffold for proliferation, remodeling, and regeneration of a variety of host tissues including blood vessels^[10], dura mater^[11], urinary bladder^[12], abdominal wall^[13], and tendons^[14]. Grossly and microscopically the remodeled tissue resembles the native tissue. Recently, SIS has been evaluated for its potential *in vivo* use in hepatocyte transplantation^[15]. For these reasons, in this study we have investigated whether the use of SIS in co-culture with rat islets could improve islet survival and their *in vitro* function.

MATERIALS AND METHODS

Animals

Male Wistar rats (250–400 g body weight, Animal Laboratory of Xi'an Jiaotong University, China) were used for islet isolation. All animals received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health guidelines.

Design of study

Islets were isolated using a collagenase and purified by discontinuous Euroficoll gradient. The purified islets were

separated into study group and control group. In study group, islets were cultured in plates coated with SIS. In control group, islets were cultured in plates not coated with SIS. The number of islets was counted after isolation and culture. Recovery of islets was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation. Islet function was determined by a static glucose challenge test after a short period of culture for 7 d and a long period of culture for 14 d. Glucose challenge test was performed in the absence and presence of IBMX. Apoptosis of islet cells following culture was measured by relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of cells by ELISA.

Preparation of soluble SIS supplement

SIS was prepared as previously described^[10]. In brief, freshly harvested porcine jejunum was obtained from a local slaughterhouse. The tube of intestinal material was rinsed, until it was free of contents and inverted. Superficial layers of the tunica mucosa were removed by scraping with a knife handle. The tissue was then reverted to its original direction, serosa and tunica muscularis were removed. The resulting membrane was approximately 80–100 μ m thick consisting of tunica submucosa and basilar portion of the tunica mucosa. The prepared intestinal submucosa tube was split open longitudinally and rinsed extensively in water to get rid of any of the cells associated with the matrix to eliminate cell degradation products. The sheets of SIS were sterilized by exposure to 1 g/L per acetic acid. To make multilayered SIS, five sheets of SIS were mechanically compressed by vacuum pressing and dried. The multilayered SIS was then terminally sterilized with ethylene oxide and kept until ready to use. Re-soaking in saline prior to use made the SIS sheets pliable and soft.

Islet isolation and culture

Islets were isolated and purified from the rat pancreas as previously described^[16]. Briefly, rats were anesthetized with intraperitoneal pentobarbital. Pancreas was infused via the common bile duct with Hanks' balanced salt solution, set apart, and minced on ice. Digestion was performed with type V collagenase (7.5 g/L; Sigma) for 25 min at 37 °C. Islets were purified on a discontinuous Euroficoll gradient (Sigma), handpicked under an inverted light microscope, pooled and then separated into study group and control group. In study group, islets were cultured in plates coated with suitable size multilayer SIS. In control group, islets were cultured in plates not coated with SIS. Islets were cultured in RPMI 1640 medium (Gibco) supplemented with 100 mL/L fetal calf serum (Gibco), 200 kU/L penicillin, 100 mg/L streptomycin, and 2 mmol/L L-glutamine (Gibco) at 37 °C in a humidified 50 mL/L CO₂ atmosphere for a period of 7 or 14 d.

Islet quantification and recovery

Islets from both groups were stained with dithizone and counted in accordance with the criteria established at

the 1989 International Workshop on Islet Assessment after isolation and culture. The number of islets was determined using an optical graticule attached to the eyepiece of a dissecting microscope and converted to the standard of islet equivalent (IE). Recovery of islets following culture was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation.

Islet viability

For each group, 6 separate samples of 20 islets each were tested simultaneously for their stimulated insulin secretion. Islets ($150 \pm 50 \mu\text{m}$ in diameter) were first pre-incubated for 45–50 min in 2 mL RPMI 1640 solution containing 2.7 mmol/L glucose. Insulin secretion was then assessed by three consecutive incubations with RPMI 1640 solution containing 2.7, 16.7, and 16.7 mmol/L glucose with 1 g/L IBMX (Sigma), each for 45 min. At the end of incubation with each solution, the supernatant was completely removed and used to test the insulin secretion level by radioimmunoassay (Department of Isotope, China Institute of Atomic Energy). Insulin secretory responses of 20 islets to glucose stimulation were expressed as milliunit per liter. To eliminate variation of islet size causing differences of insulin response, the stimulation index for each group of islets was determined by the ratio of insulin secretion stimulated by high glucose solution containing IBMX to the basal insulin secretion stimulated by low glucose solution.

Evaluation of apoptosis of islet cells

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) in culture supernatants. After 7–14 d of incubation, culture medium was collected from each well of two groups. The supernatants were analyzed to measure cytoplasmic histone-associated DNA fragments generated by cell death based on mouse monoclonal antibodies against DNA and histones, respectively. The cultured supernatant sample (20 μL) was mixed with 80 μL of immunoreagent containing two monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated) in the well coated with streptavidin of a 96-well plate. Antibody–nucleosome complexes were bound to the microplate by streptavidin. The solution mixture was incubated by gently shaking (300 r/min) for 2 h at room temperature. The solution was then collected and the well was completely rinsed thrice with 150 μL of an incubation buffer and 100 μL of premixed 2, 2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) substrate solution was added to each well. The mixed solution was agitated on a plate shaker for 20 min at room temperature and the solution absorbance was measured at a wavelength of 405 nm. The enrichment of nucleosomes due to apoptosis of islet cells was calculated from these absorbance values by the following formula: enrichment factor of nucleosomes = (values of the sample – the background values) / values of the negative control. The assay was done independently thrice for

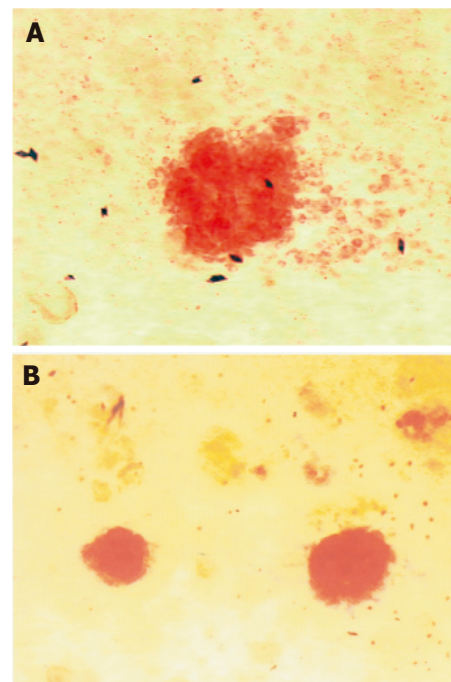


Figure 1 Morphology of islets after culture. **A:** Loose appearance of islets after 14 d of culture under standard condition; **B:** excellent morphology of islets after 14 d of culture on SIS ($\times 400$).

each experimental group. This method could permit us to specifically determine mono- and oligonucleosomes released into culture supernatants accompanying apoptosis and necrosis of islet cells. Accumulation of mono- and oligonucleosomes in the cytoplasm of apoptotic and necrotic cells caused DNA degradation, occurring several hours before the breakdown of plasma membrane.

Statistical analysis

Results were expressed as mean \pm SE. Differences between the two groups were analyzed by *t*-test using SPSS10.0. $P < 0.05$ was considered statistically significant.

RESULTS

After 7 and 14 d of *in vitro* tissue culture, SIS-treated group indicated a significantly higher ($P < 0.05$) recovery of islets than control group (Table 1). The recovery in SIS-treated group was about twice more than that in control group. In SIS-treated group, there was no statistical difference in the recovery between the short and the long periods of culture ($95.8 \pm 1.0\%$ vs $90.8 \pm 1.5\%$, $P < 0.05$). Most islets cultured in control group lost their initial morphology, becoming “loose” in appearance at the end of culture. Islets cultured with SIS exhibited excellent morphology (Figure 1).

In vitro viability of the islets was assessed using the glucose challenge test. There was no statistical difference in the basal level of insulin secretion between SIS treated group and control group ($9.1 \pm 0.3 \text{ mU/L}$ vs $7.8 \pm 0.6 \text{ mU/L}$ after 7 d of culture, $5.9 \pm 0.4 \text{ mU/L}$ vs $7.1 \pm 0.4 \text{ mU/L}$ after

Table 1 Recovery of rat islets and enrichment factor of nucleosomes after being cultured for 7 and 14 d ($n = 6$, mean \pm SE)

Parameter	Group	7 d (%)	14 d (%)
Recovery	Standard culture	67.1 \pm 2.6	42.2 \pm 1.5
	SIS culture	95.8 \pm 1.0 ^a	90.8 \pm 1.5 ^a
Enrichment factor	Standard culture	1.98 \pm 0.06	2.90 \pm 0.23
	SIS culture	1.08 \pm 0.02 ^a	1.12 \pm 0.09 ^a

^a $P < 0.05$ vs standard culture.

14 d of culture, $P > 0.05$). There was a two- to threefold increase in insulin secretion in both groups upon stimulation by high glucose solution (16.7 mmol/L). After 14 d of culture, SIS-treated group showed a significantly higher increase in insulin secretion than control group (20.7 \pm 1.1 mU/L vs 11.8 \pm 1.1 mU/L, $P < 0.05$). When islets were placed in the high glucose solution containing IBMX, the stimulated insulin secretion was significantly higher in SIS-treated group than in control group. Similar results occurred when the stimulation index was used as a reference (a ratio of insulin secreted upon stimulation by high glucose solution plus IBMX to basal insulin secreted). The stimulation index of SIS-treated group was about 2-3 times than that of the control group. In addition, the stimulation index of SIS-treated group were statistically equivalent to that of the group cultured for 7 and 14 d group (9.5 \pm 0.2 vs 10.2 \pm 1.2, $P > 0.05$) (Table 2).

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) in culture supernatants using ELISA. The enrichment factor of nucleosomes was significantly lower in SIS-treated group than in control group, suggesting that much less apoptosis of islet cells occurred in SIS-treated group (Table 1).

DISCUSSION

Islet transplantation is a viable option of treatment for insulin-dependent diabetes mellitus. Maintaining islets in culture is important to increase the safety, practicality and efficacy of successful clinical results. In addition to reducing the immunogenicity of islets^[3,17], islet culture simplifies the procedure of transplantation, which can be scheduled during regular hospital hours, and the patient is not necessary to move from a distant location to the transplantation center before a donor is available. Culture may facilitate novel immunosuppressive techniques^[18]. Moreover, improved methods and supplementation of islet culture provide a unique opportunity to optimize recovery of islet mass for transplantation. Islets do not form monolayers, fail to replicate and contain multiple cell types, making them similar to a tiny organ. All these are the unique challenges for maintaining islets in culture. Procedures related to isolation and *in vitro* culture can lead to a considerable loss of islet tissue. Apoptosis and anoikis may be the mechanisms involved in tissue loss^[19,20].

It has been reported that binding of ECM to integrin, a

Table 2 Stimulated insulin secretion of rat islets cultured for 7 and 14 d during static incubation assay ($n = 6$, mean \pm SD)

Group	T (d)	Low glucose solution (2.7 mmol/L)	High glucose solution (16.7 mmol/L)	High glucose solution +50 μ mol/L IBMX	SI ¹
Standard culture	7	9.1 \pm 0.3	18.3 \pm 1.1	51.3 \pm 3.4	5.7 \pm 0.4
SIS culture	7	7.8 \pm 0.6	21.8 \pm 1.9	75.8 \pm 2.1 ^a	10.2 \pm 1.2 ^a
Standard culture	14	5.9 \pm 0.4	11.8 \pm 1.1	20.5 \pm 1.8	3.6 \pm 0.4
SIS culture	14	7.1 \pm 0.4	20.7 \pm 1.1 ^a	66.9 \pm 3.7 ^a	9.5 \pm 0.2 ^a

¹SI (stimulation index)=insulin secretion during incubation with 16.7 mmol/L high glucose solution+50 μ mol/L IBMX/insulin secretion during incubation with 2.7 mmol/L low glucose solution. ^a $P < 0.05$ vs standard culture.

protein located on cell membrane, stimulates intracellular signaling pathways to prevent cells from their entry into the cascade of apoptosis^[21], suggesting that interaction between cells and ECM plays an important role in the subsequent cell behaviors. It is possible that cell-ECM interaction is disrupted by the procedures related to islet isolation and purification. As a consequence, functions of isolated islets could be damaged. A recent study showed that the viability of porcine islet cells embedded in fibrous skeleton of pancreas is higher viability which may be due to cellular adhesion and ligation of ECM to integrins as opposed to their isolated counterparts. Further studies of these embedded cells demonstrated that the viability of the cells is sustained for 30 days or more and islet function is excellent 30 days after the isolation^[22].

Frisch *et al.*^[23] and Thomas *et al.*^[22] have shown that anoikis resulted from a death signal induced by MEKK-1 group of caspases^[23,24]. As a critical safeguard of nature, anoikis can prevent cells from taking up residence and growth in ectopic positions as in tumor metastases. Thomas *et al.*^[22] reported that the apoptotic rate is markedly reduced when cells are attached to ECM, suggesting that ECM plays an essential role in the maintenance of differentiated cells. Preparation of islet isolation especially enzymatic digestion of pancreas can result in the loss of peripheral basement membrane or interstitial membrane of islets, suggesting that purity is not essential to achieve engraftment. The presence of other cellular elements may be critical for engraftment and long-term maintenance of graft function and survival^[25]. The fact that transplantation of purified preparations of autologous islets loses islet function in early periods also supports this hypothesis^[26].

A number of novel approaches have been developed to create a favorable physiological microenvironment for islets in culture to limit anoikis. The major method is to use ECM. Studies indicate that ECM can increase islet survival time and/or insulin secretion^[21,27,28]. As a natural ECM, SIS is obtained from the intestine using a process that retains the natural composition and configuration of matrix molecules such as collagen (Types I, III and VI), glycosaminoglycans (hyaluronic acid, chondroitin sulfate A and B, heparin and heparin sulfate), proteoglycans,

glycoproteins (fibronectin), and growth factors (FGF-2, TGF- β , and VEGF), which play an important role in host tissue repair, remodel, and cell growth^[8]. Mapping of the distribution of significant proteins and proteoglycans in small intestinal submucosa by fluorescence microscopy indicates that heparan sulfate proteoglycans (HSPG) is extensively distributed but concentrated in vessels. FGF-2 is diffusely distributed and associated with fibrous structures. VEGF is distributed mainly around the vessels. Collagen fibrils are distinctly present in the background. This anatomic structure of SIS is likely to play an important role in the regeneration of tissues and factors in remnant vessels may facilitate penetration of the matrix along these avenues^[29].

SIS has excellent mechanical properties (high compliance, a high burst pressure point, and an effective porosity index), satisfactory histocompatibility, non-immunogenicity and safety for human use, allowing it to be used for vascular and connective tissue graft construct^[7,10-14]. The porous nature and three-dimensional microarchitecture of SIS allow diffusion of cell nutrients and induce proliferation, remodeling and regeneration of host tissues when implanted in microenvironments *in vivo* (e.g. artery, tendon, bone, and articular cartilage)^[10,14,30,31]. The same situation occurs in the cells during *in vitro* culture. In a recent study, several cell lines, including NIH Swiss mouse 3T3 fibroblasts, NIH 3T3/J2 fibroblasts, primary human fibroblasts, primary human keratinocytes, human microvascular endothelial cells (HMECs) and rat osteosarcoma (ROS) cells were cultured in the presence of sheet form SIS. All cell types showed the ability to attach and proliferate. All fibroblast cell lines and keratinocytes proliferated and/or migrated into the three-dimensional scaffold of SIS matrix. Co-culturing of NIH 3T3/J2 fibroblasts and primary human keratinocytes with SIS resulted in a distinctive spatial orientation of the two cell types. Fibroblasts populated the mid-substance of the three-dimensional matrix and keratinocytes formed an epidermal structure with rete ridge-like formation and stratification, when the composite was lifted to an air liquid interface in culture^[32]. For these reasons, we hypothesize that SIS with native sheet-like configuration can imitate the natural growth environments of islets and improve islet survival and function *in vitro*, thus decreasing loss of tissue mass during culture.

We observed a higher recovery and stimulation index of islets and a lower enrichment factor of nucleosomes in SIS-treated group than in control group. These results may prove our hypothesis and are comparable to previous studies in different cell lines^[32] and islets^[6]. In pancreas, ECM is composed of interstitial matrix and basement membrane, the latter is composed of fibronectin, laminin and collagens IV and V^[33]. These submucosal cell growth substrates provide islet cells with a collagenous matrix environment *in vitro*, resembling that in *in vivo*. We think that the unique configuration and composition of SIS may contribute to such improvements for islet culture. Firstly, the porous nature of SIS allows diffusion of cell nutrients,

thus decreasing the central cell damage of isolated islets of Langerhans^[34]. Secondly, SIS provides a substratum with a three-dimensional scaffold allowing for cell migration and spatial organization^[35]. Thirdly, abundant collagen and various growth factors activate the signaling pathways, which induce attachment, proliferation, and differentiation of the cells^[36].

In conclusion, co-culture of isolated rat islets with native sheet-like SIS can provide an ECM and possible biotrophic and growth factors, which promote the recovery and subsequent function of islets during *in vitro* tissue culture. In view of the rapid degradation of SIS *in vivo*^[37], whether SIS improves the recovery and subsequent function of islets *in vitro* and the effect of SIS on islets *in vivo* needs to be further studied.

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• LETTERS TO THE EDITOR •

Complete or partial trisomy 3 in gastro-intestinal MALT lymphomas co-occurs with aberrations at 18q21 and correlates with advanced disease stage: A study on 25 cases

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TO THE EDITOR

Taji *et al.*^[1] have reported in their study on 13 patients with gastric mucosa-associated lymphoid tissue (MALT) lymphomas an aggressive tumor course in trisomy 3 positive cases. The authors analyzed only stage I patients with classical low-grade marginal zone lymphoma of the MALT type and detected the trisomy 3 using an alpha-satellite DNA probe directed to the centromere. Their data support the observation that trisomy 3 is the most frequent cytogenetic aberration in MALT lymphomas^[2,3].

In our previous published series^[4] of 29 surgically resected gastrointestinal (GI) *t*(11;18) negative MALT

lymphomas, we have described an adverse prognostic impact of trisomy 18q21 detected by a *MALT1*-specific fluorescence *in situ* hybridization (FISH) probe, especially in the lymphomas with high grade component. We have additionally studied 25 of the previously reported GI lymphomas including 7 low grade marginal zone lymphomas and 18 GI diffuse large B-cell lymphomas (DLBCL, including 3 cases with low grade lymphoma component) diagnosed in stage I ($n = 8$) and in stage >II ($n = 17$) for complete and partial trisomies 3 using FISH probes for the centromere of chromosome 3 (Vysis, Downer's Groove, IL, USA) as well as for the *BCL6* gene at 3q27 (flanking BAC clones RP11- 528E8 and 690C8)^[5]. The latter double-color assay was selected because it targets the critically gained band of chromosome 3 in MALT lymphomas and can detect breakpoints affecting the *BCL6* locus, which are recurrent in extranodal DLBCL. For each hybridization, we evaluated 100 cell nuclei. The cut-off level, defined as mean false positive rate (determined in normal gastric mucosa and samples from *Helicobacter pylori* gastritis) plus three standard deviations, was 5.4% for the centromere 3 probe (trisomy). For the *BCL6* probe mix no split or gain of signals was observed in the control samples, but according to the literature, the cut-off level for the detection of trisomy 3q27 or *BCL6* breaks was set to 3%^[5].

In agreement with the previously published studies, we detected trisomy 3q27 as the most frequent aberration in GI MALT lymphomas, occurring in 9/25 (36%) of the cases. In three of these nine cases, trisomy 3 was indicated by the presence of supernumerary signals for both the centromeric region and the *BCL6* locus, whereas in the remaining six cases, a gain of 3q27/*BCL6* was detected without a change in the number of centromere signals (partial trisomy 3q27). The only positive stage I lymphoma showed a partial trisomy 3q27. In three cases of the DLBCL subset, one with trisomy 3 and two with partial trisomy 3q27, a separation of the *BCL6* signal pair was additionally detected indicating both a *BCL6* translocation (two cases potentially with *BCL6*/*IGH* rearrangement) and overrepresentation of proximal/distal 3q27.

Trisomies 3q27 were present in 1/8 (13%) stage I and 8/17 (47%) stage >II diseases indicating a significant ($P < 0.01$; χ^2 test) association of trisomy 3q27 with

lymphoma dissemination. There was a strong correlation between the occurrence of trisomies 18q21/*MALT1* and 3q27/*BCL6* in the present series of GI MALT lymphomas with 5/6 (83%) 18q21 positive tumors carrying both aberration and only 1/6 (17%) case showing trisomy 18q21 without trisomy 3q27 ($P < 0.05$). We have previously reported that trisomy 18q21/*MALT1* to be associated with an unfavorable prognosis in the same series of surgically resected GI MALT lymphomas, which was particularly pronounced in the DLBCL subset^[4]. With regard to trisomy 3q27, we observed a trend towards an inferior survival too, $P = 0.0727$.

Our data highlight that partial trisomies of chromosomes 3 and 18 have to be taken into account when evaluating the course of GI MALT lymphomas. Roughly two-thirds of the gains on the respective chromosomes might not include the centromeric region. Moreover, the co-occurrence of trisomies 3q27 and 18q21 in our group of surgically resected GI MALT lymphomas points to a possible interaction of both loci, especially in patients with an advanced stage disease.

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www.hepatologia.org

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September 12-14, 2005
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Original Research, Clinical Trials, Reviews, Comments, and Case Reports in esophageal cancer, gastric cancer, colon cancer, liver cancer, viral liver diseases, *etc.*, from all over the world are welcome on the condition that they have not been published previously and have not been submitted simultaneously elsewhere.

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Data which is not statistically significant should not be noted. ^a*P*<0.05, ^b*P*<0.01 (*P*>0.05 should not be noted). If there are other series of *P* values, ^c*P*<0.05 and ^d*P*<0.01 are used; Third series of *P* values can be expressed as ^e*P*<0.05 and ^f*P*<0.01. Other notes in tables or under illustrations should be expressed as ¹*F*, ²*F*, ³*F*; or some other symbols with a superscript (Arabic

numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, etc. in a certain sequence.

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- 2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]
- 3 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287 [CMFAID:1082371101835979]

Books and other monographs (list all authors)

- 4 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 5 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Electronic journal (list all authors)

- 6 **Morse SS**. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Statistical data

Present as mean±SD and mean±SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

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, *p*(CEA) = 8.6 24.5 μg/L; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; 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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Italicization

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gvrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

Biology: *Helicobacter pylori*, *H pylori*, *E coli*, etc.

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Number	Nonstandard	Standard	Notice
1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (V/V)	500 mL/L	
20	50% (m/V)	500 g/L	
21	1 M	1 mol/L	
22	10 μ M	10 μ mol/L	
23	1N HCl	1 mol/L HCl	
24	1N H ₂ SO ₄	0.5 mol/L H ₂ SO ₄	
25	4rd edition	4 th edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku, 18 500u or M:18 500	
28	25 g.kg ⁻¹ /d ⁻¹	25 g/(kg.d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg L ⁻¹	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 g	High speed; g should be in italic and suitable conversion.
35	1000 g	1 000 r/min	Low speed. g cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μ mol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD ₂₆₀	A ₂₆₀	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	A ₂₆₀ nm ^b P<0.05	A ₂₆₀ nm ^a P<0.05	A should be in italic. In Table, no note is needed if there is no significance in statistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If there is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^e P<0.05, ^f P<0.01. Notices in or under a table
45	[*] F=9.87, [§] F=25.9, [#] F=67.4	¹ F=9.87, ² F=25.9, ³ F=67.4	
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mm ³	kJ/mol	kilojoule per mole
65	10×10×10cm ³	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$\bar{x}\pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	im	im	intramuscular injection
70	iv	iv	intravenous injection
71	Wang et al	Wang et al.	
72	EcoRI	EcoRI	Eco in italic and RI in positive. Restriction endonuclease has its prescript form of writing. Bacteria and other biologic terms have their specific expression.
73	Ecoli	E.coli	
74	Hp	H pylori	
75	Iga	Iga	writing form of genes
76	igA	IgA	writing form of proteins
77	~70 kDa	~70 ku	