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EDITORIAL

Third-line rescue therapy for *Helicobacter pylori* infection

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Abstract

H pylori gastric infection is one of the most prevalent infectious diseases worldwide. The discovery that most upper gastrointestinal diseases are related to *H pylori* infection and therefore can be treated with antibiotics is an important medical advance. Currently, a first-line triple therapy based on proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) plus two antibiotics (clarithromycin and amoxicillin or nitroimidazole) is recommended by all consensus conferences and guidelines. Even with the correct use of this drug combination, infection can not be eradicated in up to 23% of patients. Therefore, several second line therapies have been recommended. A 7 d quadruple therapy based on PPI, bismuth, tetracycline and metronidazole is the more frequently accepted. However, with second-line therapy, bacterial eradication may fail in up to 40% of cases. When *H pylori* eradication is strictly indicated the choice of further treatment is controversial. Currently, a standard third-line therapy is lacking and various protocols have been proposed. Even after two consecutive failures, the most recent literature data have demonstrated that *H pylori* eradication can be achieved in almost all patients, even when antibiotic susceptibility is not tested. Different possibilities of empirical treatment exist and the available third-line strategies are herein reviewed.

Key words: *Helicobacter pylori*; Third-line rescue therapy; Antimicrobial resistance; Levofloxacin; Rifabutin; Furazolidone; Doxycycline

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a spiral-shaped bacterium that is attached to or just above the gastric mucosa. The organism can persist in the stomach indefinitely and may not cause clinical illness for many years after infection. Indeed, a large number of infected patients never develop any symptoms. However, a large body of literature has associated *H pylori* infection with gastritis and gastric malignancies (gastric adenocarcinoma and MALT-lymphoma)[1]. Chronic *H pylori* infection has also been associated with several extra intestinal diseases, such as autoimmune thrombocytopenia, sideropenic anemia and chronic urticaria but the pathogenesis is still not known [2].

H pylori gastric infection is one of the most prevalent infectious diseases worldwide with an estimation of 40%-50% of the world population. Remarkable differences are due to geographical, socio-economical and demographic factors [3, 4]. *H pylori* transmission is still not completely understood. In addition, among infected patients, the reasons why only some develop symptoms is still a matter of speculations. The more generally accepted point of view is that bacteria are likely spread from person to person by fecal or oral transmission. Humans are the primary reservoir of *H pylori* infection [5].

Several tests are available to detect *H pylori* in patients with ulcer or dyspepsia. The more commonly used tests are the evaluation of bioptic specimens during upper GI endoscopy, the detection of serum anti *H pylori* antibodies and breath tests with ^{13}C-labeled urea [6].

The discovery that most upper gastrointestinal diseases are the consequence of *H pylori* infection and can be treated with antibacterials is an important medical advance [7]. In the last few decades, *H pylori* eradication has been standardized. The occurrence of resistance to therapeutic regimens is a growing problem.

Selection of papers was based on those papers thought to be more relevant to the authors based on two criteria: larger studies and novel studies even if based on limited series of patients, in which case this limit was stated.

FIRST-LINE THERAPY

The first-line therapy protocol is now generally accepted [8-12], which consists of proton pump inhibitor (PPI) (b.i.d.) or ranitidine bismuth citrate (RBC) plus two antibiotics: clarithromycin (500 mg, b.i.d.) and amoxicillin (1 g, b.i.d.) administered for 7 d. Metronidazole (500 mg, b.i.d.) can be used as an alternative to amoxicillin. However, even with

the correct use of these drug combinations, infection is not eradicated in 10%-23% of patients [13].

FACTORS DETERMINING PRIMARY ERADICATION FAILURE

H pylori may develop resistance to the prescribed antibacterials and may acquire resistance by acquisition and recombination of genes from other bacteria [14]. Chromosomic mutations can also induce resistance [15]. Gene acquisition is unlikely because *H pylori* lives alone in a unique ecological niche and is equipped with multiple restriction systems to avoid the introduction of hexogenous DNA [16]. Therefore, resistance is generally thought to be the consequence of point mutations. Indeed metronidazole targets DNA and a high mutation rate is observed [15].

After the development of eradication therapies, *H pylori* resistant strains have rapidly disseminated [17-21]. Several mechanisms are involved in the development of resistance. First, the lack of patient compliance is assumed to be a key factor in eradication failure, which occurs because adverse events are relatively frequent and lead to treatment discontinuation [22, 23]. Second, insufficient antibiotic concentration at the site of infection contributes to the spreading of resistant strains [22, 23].

An emerging problem is that general practitioners prescribe treatments without adequate diagnosis and do not adhere to eradication guidelines [24, 25]. Given the importance of host immune response in *H pylori* infection, the role of immunity in eradication failure can be hardly argued. However, data are anecdotal. Borody *et al* [26] suggested that IL-4 is important in *H pylori* eradication and hypothesized that IL-4 defect contributes to eradication failure.

Cytochrome P450, isoenzyme 2C19 [27] and interleukin-1-beta polymorphisms can interfere with acid secretion and have the activity of antimicrobial agents [28].

Finally, socio-economic factors (smoking habit), geographical factors, gender, histological changes also affect the eradication success [23-25]. Disease phenotypes also contribute to eradication failure. In fact, the failure rate in duodenal ulcer is 21.9%, lesser than in nonulcer dyspepsia (33.7%). In addition, the presence of histological fibrosis and lympho-epithelial lesions leads to poor eradication rates [23, 29].

Large studies on all these possible mechanisms of failure are lacking, but clarithromycin resistance appears to be the most important mechanism [30, 31].

SECOND-LINE THERAPY

Second-line therapy has been extensively reviewed by several authors[30, 32-34]. Therefore, we herein only discuss the Maastricht guidelines and some of more recently proposed protocols using new antimicrobial drugs, such as levofloxacin, rifabutin and furazolidone.

Most authors concord that culture after a first eradication failure is not thought to be necessary to start the second-line therapy. The assessment of *H pylori* sensitivity to antibiotics may be useful only after failure of the second-line therapy [8, 9, 35]. As second-line therapy, the Maastricht 2-2000 Consensus Report suggests a quadruple therapy based on bismuth (120 mg, q.i.d.), tetracycline (500 mg, q.i.d.), metronidazole (500 mg, t.i.d.) and antisecretive agent (PPI, b.i.d.) for a minimum of 7 d [12].

Further trials have shown that replacing the proton pump inhibitor and the bismuth compound of the quadruple therapy by RBC also achieves good results, with an eradication rate ranging between 57%-95% [36-39]. The failure of second line quadruple therapy is associated with its discontinuation because of the high incidence of side effects (6%-68%) [40]. Low compliance for the high number of pills to be taken each day also affects the clinical results [24]. However, in second-line regimens, new combination of drugs has been used. A triple therapy with the combination of levofloxacin, rabeprazole and tinidazole or amoxicillin has been proposed as an alternative to Maastricht [41]. This protocol shows an eradication rate higher than 90% compared to quadruple therapies given for 7 d (63%) with a lower incidence of side effects [42].

Rifabutin has been shown to have a good eradication rate (87%), if administered at a high dose (300 mg) in combination with amoxicillin and PPI, as compared to quadruple therapy [43-47]. Rifabutin shows an important side effect (mielotoxicity) [46]. Wong *et al* [43] showed that a combination of levofloxacin, rifabutin and rabeprazole has a high efficacy with an eradication rate >90% [43].

Furazolidone is also used to replace metronidazole in quadruple therapy [48-51]. Different *in vivo* studies have confirmed the efficacy of regimens containing a high-dose furazolidone [200 mg, b.i.d.] as the second-line therapy in patients with metronidazole-resistance [48-51]. Many other combinations have been used [31] with various rates of success. Bacterial eradication may fail in up to 40% of cases after the suggested second-line regimens. As a consequence, to treat patients who have already undergone the first- and second-line therapies is a common challenge.

THIRD-LINE RESCUE THERAPY

Currently, a standard third-line therapy is lacking. Different groups have tested various therapeutic protocols [33, 52, 53]. When available, endoscopy with culture and consequent antibiotic susceptibility testing remains the most appropriate option for patients with two eradication failures [54-56] to avoid a widespread use of expensive antibiotics such as rifabutin. The use of these drugs may also induce severe side-effects and development of *H pylori* resistant strains [30]. However, systematic use of culture is questionable [57]. Culture implies general endoscopic risks and is expensive as well as time-consuming due to *H pylori* difficult growth and not always available on a routine basis [58].

The sensitivity of bacterial culture is not 100% even in expert hands [6]. Moreover, amoxicillin and tetracycline rarely induce resistance [58, 59]. On the other hand, most of *H pylori* isolates after two eradication failures are resistant to metronidazole and clarithromycin, respectively [55]. Therefore, these two drugs are not recommended for third-line therapy [22, 56]. Our own previous data also show high resistance rates to metronidazole and clarithromycin even if the previously used regimens did not include either

of these two drugs [55]. In addition, *in vitro* susceptibility cannot predict eradication success [60-62]. Taken together, these data suggest that cultures are not strictly necessary to decide upon a third-line protocol.

The third-line therapy should avoid metronidazole and clarithromycin and antibiotics that are likely to have contributed to development of resistance. A consensus for third-line therapies has not been presently reached. Herein we discuss those based on levofloxacin, rifabutin, furazolidone and doxycycline.

Levofloxacin-based therapy

Levofloxacin is a broad-spectrum fluoroquinolone, active against Gram-positive and negative bacteria and atypical respiratory pathogens [63]. Levofloxacin inhibits the DNA synthesis, has a good oral absorption and is well tolerated [64]. Fluoroquinolones are active against *H pylori in vitro* [65] and have a synergistic effect with PPIs [66]. Primary resistance to levofloxacin ranges between 8%-31% in different countries or regions [55, 67, 68].

Recently, Gatta *et al* [69] have proposed a third-line treatment after two eradication failed courses without fluoroquinolones, with standard dose of PPIs (b.i.d.), levofloxacin (250 mg, b.i.d.) and amoxicillin (1 g, b.i.d.) for 10 d. The eradications rates of 76.2% and 84.6% according to ITT and PP analysis, respectively, have been achieved in 151 enrolled patients in a prospective open study. The levofloxacin-based treatment could eradicate most of the strains (92.3%) which are resistant *in vitro* to both clarithromycin and metronidazole, but susceptible to levofloxacin. The primary resistance to levofloxacin found in this study was 14%. Furthermore, this drug combination, successfully employed as rescue therapy [70], is well tolerated and has no major side-effects [71].

A more recent prospective multicentric study [72] reports data of 100 patients who have failed two eradication courses without fluoroquinolones. This study demonstrated that a regimen of levofloxacin (500 mg, b.i.d.), amoxicillin (1 g, b.i.d.) and omeprazole (20 mg, b.i.d.) for 10 d can achieve an eradication rate of 60% or 66% according to ITT and PP analysis. The treatment was given without previous sensitivity test.

Low compliance with the current regimens is one of the main causes of failures [24]. Therefore, Coelho *et al* [73] have proposed a combination of rabeprazole (20 mg), levofloxacin (500 mg) and furazolidone (200 mg) (two tablets) administered at a single dose for 10 d. Twelve patients who failed at least two eradication courses are successfully treated. Per-protocol and intention-to-treat eradication rates were 100% and 83.3%, respectively. However, because of the paucity of patients in third-line therapy, these data have to be confirmed in larger series. Furthermore, cultures obtained before treatment from some patients show no resistance to furazolidone, while 87% of the samples analyzed are sensitive to levofloxacin. No severe adverse effects are observed [73]. Therefore, the results after the levofloxacin-based triple therapy for ten days in patients with two eradication failed courses with amoxicillin, clarithromycin, metronidazole, tetracycline and bismuth, are encouraging. However, the resistance to quinolones is easily acquired, and the resistance rate is relatively high in countries with a high consumption of these drugs [55, 68]. Therefore, it seems advisable to reserve levofloxacin to third-line rescue treatment to avoid the increase of the resistance phenomenon [72].

Rifabutin-based therapy

Rifabutin is a spiropiperidyl derivative of rifamycin-S, an antitubercolar compound. Rifabutin inhibits the beta-subunit of *H pylori* DNA-dependent RNA polymerase encoded by the rpoB gene [74]. Rifabutin is expensive and unavailable in various countries and has side effects (leukopenia and thrombocytopenia, with myelotoxicity) [46]. It has been suggested to reserve the use of rifabutin for the treatment of multiresistant *Mycobacterium tuberculosis* strains [53, 75]. *H pylori* is highly susceptible *in vitro* to rifabutin and no resistant strains have been isolated from patients treated or untreated for *H pylori* infection [74, 75]. Furthermore, rifabutin is chemically stable at a wide pH range [76].

Three different trials have shown that rifabutin (300 mg o.d. or 150 mg b.i.d)-based therapies in combination with amoxicillin (1 g, b.i.d.) and standard dose of PPIs (b.i.d.) are a good third-line strategy, achieving the eradication rate of at least 70% [46, 47, 53]. On the other hand, Qasim *et al* [77] have achieved only a 38% eradication rate [77].

A more recently single centre prospective study [78] studied 67 patients who failed to respond to two or more courses. The result showed that when the rifabutin dose is reduced from 300 mg to 150 mg, it results in a signficant drop in eradication rate from 86.6% to 66.6% [45]. Borody *et al* [78] have shown that a 12 d regimen with low-dose rifabutin (150 mg a day) in combination with increased frequency of amoxicillin (1 g, t.d.s.) and pantoprazole (80 mg, t.d.s.) could achieve an overall eradication rate of 92.1% in patients harbouring double resistance strains to metronidazole and clarithromycin, with an eradication rate of 95.7%. Mild side effects are found in 40% of patients. Unlike regimens which use higher doses of rifabutin, no patients develop drug-related neutropenia or thrombocytopenia after treatment. Nevertheless, the main problem with a widespread use of rifabutin is the concern that antibiotic resistance may develop against *Mycobacterium avium* in HIV-infected patients. Therefore, the use of this drug for *H pylori* is questionable.

Furazolidone-based therapy

Furazolidone is a broad-spectrum nitrofuran, active against Gram-negative and positive bacteria and protozoa by inhibiting bacterial enzymes [79]. It is widely used in low income populations because it is inexpensive. It kills *H pylori* [80,81]. Strains resistant to furazolidone are rare [82, 83] and its potential to develop resistance is as low as bismuth compounds or amoxicillin [84]. Furthermore, it has no cross-resistance to metronidazole [83] and is effective in populations with a high prevalence of metronidazole resistance [85]. It has poor oral absorption and presents some side effects, especially gastrointestinal ones [79]. Concomitant intake of alcohol and MAO-inhibitors should be avoided as other interacting drugs. Furazolidone may induce a disulfiram-like reaction to alcohol and is an MAO-inhibitor. One week quadruple regimen with lansoprazole (30 mg, b.i.d), bismuth (240 mg, b.i.d.), tetracycline (1g, b.i.d.) and fura-

zolidone 200 mg (b.i.d.) has shown an eradication rate of 90% as third-line therapy in 10 patients with metronidazole resistance by culture [48]. Furthermore, 7 d triple-regimen comprising of furazolidone (200 mg, b.i.d), amoxicilin 1 g (b.i.d.) and standard dose of PPI (b.i.d.), achieves an eradication rate of 60% in 10 patients who failed first-line, second-line and rifabutin-based triple therapy [77].

In conclusion, in developing countries where resistance to metronidazole is usually very high [12], furazolidone in combination with tetracycline, bismuth and PPI for one week is very effective, safe and cost effective against *H pylori* as the third-line therapy.

Doxycycline-based therapy

Doxycycline is a widely used tetracycline antibiotic for several infections. With respect to tetracycline, doxycycline requires the administration of only two tablets per day, leading to a better compliance in patients undergoing eradication therapies. Furthermore, Heep *et al* [19] have found no secondary resistance to doxycycline in *H pylori* isolates from patients who failed one or more eradication therapies.

Quadruple regimens represent the most widely used rescue therapy. Yet, it is limited by lack of patient compliance due to the large number of tablets and by several side-effects. The classic quadruple therapy includes bismuth salts which have a synergistic effect on antibiotics possibly by decreasing the bacterial load, PPI which facilitates antibiotic activity by increasing the gastric pH, tetracycline with a low rate of resistance in *H pylori* isolates, and metronidazole [58, 59]. Induction of metronidazole resistance has suggested a new protocol, namely replacing tetracycline with doxycycline (because it requires the administration of only two tablets per day) and metronidazole with amoxicillin (because its resistance is less 1%), 1-week-quadruple therapy with doxycycline (100 mg, b.i.d.), amoxicillin (1 g, b.i.d.), omeprazole (20 mg, b.i.d.) and bismuth salts (120 mg, two tablets b.i.d). This treatment has proved to be a highly effective third-line 'rescue' therapy, achieving 91% eradication rate in patients harbouring metronidazole and clarithromycin resistant *H pylori* strains (by ITT analysis) [55]. This regimen, showing excellent compliance (99%) and mild side-effects, may well constitute the test available option for the third-line rescue treatment.

Rifampicin-based therapy

Rifampicin is a semisynthetic derivative of rifamycin B. The target is the DNA-dependent DNA polymerase, mainly the beta subunit [86]. Rifampicin inhibits the growth of most Gram-positive and negative microorganisms. The clinical efficacy of rifampicin against *H pylori* has been discovered by the observation of the decrease of anti-*H pylori* antibodies in patients on rifampicin-containing antitubercular therapy [87]. Rifampicin has an excellent *in vitro* efficacy against *H pylori* [88, 89] and a favorable pharmacokinetics. Less-expensive rifabutin is available in many countries. A single-center study has shown that 10 d rifampicin (450 mg o.d.)-triple therapy in combination with esomeprazole (40 mg b.i.d.) and tetracycline (1 000 mg b.i.d.) can achieve an eradication rate of 32.1% and 31.6% (by ITT analysis), if given as second-line or third-line therapy, respectively. Side effects are common but minor.

In conclusion, rifampicin-based rescue therapy is not as effective as a salvage-based therapy for *H pylori* eradication [86].

CONCLUSION

An undisputed third line strategy to cure *Helicobacter pylori* is still lacking. Eradication rates >90% can be achieved following the Maastricht guidelines for first- and second-line therapies. New first-line alternative strategies are needed, considering the development of primary and secondary resistances. Second-line therapy depends on which regimen is used initially, the re-administration of any antibiotics against which *H pylori* has probably become resistant, as metronidazole and clarithromycin or drugs with cross-resistance to these or previously used antimicrobial are not recommended. To face treatment failures, several third-line 'rescue' therapies have been tested, achieving good eradication rates. In our opinion, levofloxacin-triple (eradication rate of 92%) [69] and doxycycline-quadruple (eradication rate of 91%) [55] are more active on resistant strains. They are safe, better tolerated and less expensive than rifabutin-based regimen. Moreover, the widespread use of rifabutin may be a major concern due to the possible development of antibiotic resistance. We believe that the worldwide aid tubercular emergency and the risk to develop *Mycobacterium*-resistant strains strongly suggest a conservative approach reserving rifabutin to antitubercular therapy. This is especially recommended in countries where alternative drugs are available. In developing countries where resistance to metronidazole is usually very high, the 7 d furazolidone-quadruple third-line therapy is effective against *H pylori* (with eradication rates of 90%), safe and cost-effective.

In conclusion, our review shows that *Helicobacter pylori* eradication can be eventually obtained even in the few patients who experience up to 8 consecutive failures [78, 90, 91]. This can be done by different drugs as reported in the different protocols discussed above.

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EDITORIAL

Pathogenesis of primary biliary cirrhosis: A unifying model

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Abstract

Primary biliary cirrhosis (PBC) is a disease of unknown etiology leading to progressive destruction of small intrahepatic bile ducts and eventually to liver cirrhosis and failure. It is characterised by female predominance and serum auto-antibodies to mitochondrial antigens targeting the E2 components of the 2-oxoacid dehydrogenase complex. Although they are associated with disease pathogenesis, no concrete evidence has been presented so far. Epidemiological data indicate that a geographical clustering of cases and possible environmental factors are implicated in pathogenesis. A number of genetic factors play a role in determining disease susceptibility or progression, although no definitive conclusion has been reached so far. A key factor to immune pathogenesis is considered to be the breakdown of immune tolerance, either through molecular mimicry or through the so called determinant density model. In this review, the available data regarding the pathogenesis of primary biliary cirrhosis are described and discussed. A new unifying hypothesis based on early endothelin overproduction in primary biliary cirrhosis (PBC) is presented and discussed.

Key words: Primary biliary cirrhosis; Pathogenesis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease of unknown aetiology characterized by progressive destruction of small intrahepatic bile ducts eventually leading to cirrhosis. It is considered to be an autoimmune disease due mostly to the presence of well characterized auto-antibodies. These auto-antibodies target the components of 2-oxoacid dehydrogenase complexes. Antibodies against components of the nuclear pore complex have also been described. The disease may be considered as an example of the vanishing bile duct syndrome.

Although auto-antigens have been molecularly identified and epitope-mapped and auto-reactive T and B cells have been characterized, the exact mechanism of liver tissue damage remains unclear. Recent reviews have summarised the present theories of PBC pathogenesis [1,2]. In this review, we first examined the evidence and then the current models concerning disease pathogenesis. Finally, a unifying hypothesis based on recent observations is proposed.

PRIMARY BILIARY CIRRHOSIS AS A GENETIC AND ENVIRONMENTAL DISEASE

Following earlier case reports of familial cases of PBC, a more comprehensive cohort study has estimated the sibling relative risk for PBC at 10.5, similar to other classical autoimmune diseases [3]. Recently, a pairwise concordance rate of 0.63 for PBC in monozygotic twin pairs has been published [4], which is one of the highest reported in autoimmunity. Taken together these reports indicate a significant genetic contribution to the disease pathogenesis. However, studies on specific genes have provided only weak associations. Extensive reviews on genetic factors in PBC have been recently published [5, 6].

Geographical disease clusters have been reported [7-9] and provide evidence for an as yet unidentified environmental factor in PBC pathogenesis. Although these studies are criticized and the case control studies are not able to identify putative environmental factors [10,11], there is enough evidence that environmental susceptibility does play a role.

PRIMARY BILIARY CIRRHOSIS AS A HUMORAL IMMUNE RESPONSE DISEASE

High titers of antibodies against mitochondrial elements are characteristic of the disease. Anti-mitochondrial antibodies (AMA) target the E2 component of the

pyrurate dehydrogenase complex (PDC-E2), which belongs to the family of the 2-oxoacid dehydrogenase complexes (2-OADC) [12]. The main epitopes have been localised within the inner lipoyl-binding domain of the subunit overlapping amino acids 212-226. The AMA response is polyclonal and these antibodies also react with the dihydrolipoamide dehydrogenase binding protein [13].

Based on the in vitro fact that 2-OADC activity is inhibited by AMA and 10% of the portal B cells produce antibodies reactive with PDC [14,15], a pathogenetic role of AMA has been proposed.

Sera from over 50% of patients do contain AMA with a different specificity. They react with the E1α component of PDC [16]. Their reactivity is directed to the C-terminus of the molecule which contains the active site of the enzyme and therefore these antibodies are also inhibitory of PDC activity [17,18]. Antibodies against the branched chain of 2-oxoacid dehydrogenase complex E1α have also been identified [19]. Auto antibodies against the nuclear pore proteins gp210 and p62 are associated with more active or severe disease [20]. Perhaps the best evidence for the pathogenetic role of auto antibodies in PBC comes from the description of secretory IgA anti-PDC in saliva, bile and urine of patients which retain their enzyme inhibitory capacity [21-24].

In PBC, both biliary epithelial cells and salivary epithelial cells (the main targets of the disease process) demonstrate an apical surface up-regulation of PDC or an antigen cross reacting with it [25]. This expression appears earlier than the reported up-regulation of other surface molecules like MHC class II, or ICAM-1 [26]. It seems, however, that PDC is released from apoptotic mitochondria to the cytoplasm within six hours of the induction of apoptosis and that auto-reactive epitopes are present on the still intact cell surface at later time points during the process of apoptosis [27]. However, convincing evidence for a role of AMA in the pathogenesis of PBC has yet to be produced [28]. Moreover, the very existence of the so called autoimmune cholangitis or AMA-negative PBC, a disease similar in every aspect to PBC but without detectable AMA, strongly argues against a pathogenetic role of AMA.

T CELL RESPONSES IN PBC

CD4 and CD8 T-cells reactive with PDC have been identified in the peripheral blood and liver of PBC patients [29-31]. These cells are reactive with the native human antigen [32, 33]. PDC- E2 specific T-cells are present in the liver of PBC patients [29, 34], mostly during the earliest disease states [30, 31]. Epitope mapping studies have identified HLA DR4*0101-restricted T cell epitope, spanning residues 163-176 of PDC-E2 [35, 36]. Recently HLA-A2-restricted CD8 T cell lines reactive with PDC-E2 residues 159-167 have been characterised [37, 38]. Interestingly, CD8 T cells from livers of PBC patients demonstrate cytotoxicity against PDC-E2 159-167 pulsed autologous cells [39].

APOPTOSIS IN PBC

There is concrete evidence that apoptosis is possibly the most important mechanism of biliary epithelial cell loss. Markers of ongoing apoptosis have been reported within affected portal tracts [40,41], including down regulation of the anti-apoptotic protein bcl-2 [42]. Apoptosis is considered the result of the attack of effector cells like CD8 T cells [39]. Interestingly, *in vitro* caspase cleavage of PDC-E2 has been shown to generate immunologically active protein fragments [43].

ROLE OF REACTIVE OXYGEN SPECIES (ROS) in PBC

Data on the role of oxidative stress in the pathogenesis of PBC are scarce. In the damaged bile ducts of PBC, glutathione-S-transferase expression is markedly reduced, reflecting reduction of intracellular glutathione, while perinuclear expression of 4-hydroxynonenal is increased, reflecting active lipid peroxidation associated with biliary epithelial damages [44]. Levels of the antioxidant vitamin E have been found to be decreased in PBC, together with other fat soluble vitamins [45-47], while serum total antioxidant capacity (measured with an enhanced chemiluminescent technique) is significantly reduced in PBC patients [48].

A number of antioxidant substances including retinol, alpha-tocopherol, total carotenoids, lutein, zeaxanthin, lycopene, alpha and beta-carotene are reduced in PBC patients compared to normal controls [49]. However, we have reported highly corrected total antioxidant capacity in PBC [50], a fact that may reflect a compensatory but probably not sufficient increase to counteract an increased ROS production.

Evidence for a role of ROS in the liver damage of PBC is provided by *in vitro* reports that ursodeoxycholic acid (UDCA), a drug commonly used in PBC, has extensive ROS scavenging properties and prevents mitochondrial oxidative stress and lipid peroxidation in a dose-dependent manner [51-53]. Finally, evidence from the rat bile duct-ligated model may have relevance to PBC.

Lipid peroxidation is a relatively late event in this model and a close link seems to exist between lipid peroxidation and the activation of inflammatory cells [54,55]. Free radicals triggering hepatic injury in this model, involve overproduction of the pro-inflammatory cytokines TNFα, IL-6 and IL-1b via enhanced activation of nuclear factor kB [56]. Moreover, *in vitro* experiments have shown that several bile acids including taurochenodeoxycholic acid and taurocholic acid cause hepatocyte injury with a concomitant generation of hydroperoxide by mitochondria [57,58] and also induce hepatocyte apoptosis in a time- and concentration-dependent manner via ROS generation by mitochondria [59]. An increased bile acid concentration is a feature of at least late PBC.

CURRENT VIEWS ON THE PATHOGENESIS OF PBC

There are two fundamental facts that should be interpreted in every model trying to explain the pathogenesis of PBC. First, the PBC auto-antigen PDC is located on the inner surface of the inner mitochondrial membrane and is therefore normally separated from the extra-cellular immune system by three membranes. It is difficult to understand how such an antigen is exposed to antigen presenting cells, eliciting an autoimmune reaction. Second, PBC is a disease with very limited tissue distribution, yet the putative autoimmune response is directed at an antigen with an extremely widespread localization.

So far, the models developed to explain the pathogenesis of PBC suggest that the key step in disease pathogenesis is the breakdown of T cell self-tolerance to PDC, since the induction of anti-PDC antibodies is not enough by themselves to produce liver disease [60]. The mechanisms of the disease pathogenesis have been elegantly reviewed elsewhere [61].

Molecular mimicry model of self-tolerance breakdown

Infection, either viral or bacterial, can either directly induce apoptosis of biliary epithelial cells or more probably trigger an immune attack on epithelial cells as a result of molecular mimicry. A T-cell response is initiated and mediated by toll-like receptor interaction with a pathogen epitope cross-reactive with a self-PDC epitope. An immune attack on biliary epithelial cells is mediated by these T-cells leading to apoptosis. However, the evidence for the initiating micro-organism is conflicting. Studies implicating mycobacteria as the source of cross-reactive targets are not reproducible and recent reports on Chlamydia pneumoniae as the potential microbial factor require confirmation [62].

Non PDC-E2 microbial sequences with a high degree of similarity to PDC-E2 212-226 epitope, mostly *E coli* mimics, are described as the major targets of cross-reactivity with human PDC in the sera of PBC patients [63]. Recently, the cross-reactive target has been reported to be the myobacterial hsp65 sharing a common motif with PDC-E2 212-226 epitope [64]. IG G3 antibodies to mimics from Lactobacillus delbrueckii with the same motif cross-reactive target can react with the PDC-E2 212-226 epitope in PBC sera [65]. Therefore, this motif may be a candidate epitope in the molecular mimicry model.

An alternative explanation for the molecular mimicry model would be a retroviral infection. The retroviral etiology of PBC has been recently reviewed in detail [28], but still remains controversial [66].

Determinant density model

This model has been described in detail by Jones [1]. According to this model, potentially self-PDC reactive T cells survive negative selection in the thymus, because their T cell receptor (TCR) shows low affinity for the complex of self-peptide and MHC. Sporadic self-PDC-derived epitopes presented by antigen presenting cells (APC) to these low TCR affinity T-cells, are unable to activate T cells. However, enrichment of APC presentation of self-PDC-derived epitopes could give sufficient low affinity presentation to overcome a triggering threshold and induce a proper CD4 T-cell activation.

In this model also, the initial trigger of antibody response cross-reaction with self-PDC could be either viral or bacterial epitopes with a structural homology to PDC. An interesting feature of this model is that the state of activation of APC mediated through toll-like receptors may determine the efficacy of antigen presentation and promote tolerance breakdown [67]. Peripheral blood monocytes from PBC patients produce higher levels of pro-inflammatory cytokines (TNFα, IL1b, IL-6, IL-8) when they are challenged with specific ligands for TLR2, TLR3, TLR4, TLR5 and TLR9. These findings indicate that monocytes in PBC (and possibly APC) are hyper-responsive to signalling through TLRs, a fact that may help in tolerance breakdown.

PATHOGENESIS OF PRIMARY BILIARY CIRRHOSIS: A UNIFYING HYPOTHESIS

We recently reported a significant increase of endothelins, particularly ET2 (and to a lesser extent of ET1) both in peripheral blood and in the hepatic vein, occurring at an early stage of the disease. Moreover, UDCA treatment caused a significant reduction of all three endothelins, its effect being most pronounced in early stage PBC.

Based on our observations, a new unifying hypothesis for the pathogenesis of PBC is proposed (Figure 1). In this model, there is a primary dysfunction of endothelial cells overproducing ET-2 (and to a lesser extent ET-1). This could be a primary genetically determined event. Endothelial cells express scavenger receptor type B [68] and internalise foreign antigens. Indeed lipoteicholic acid, a strongly antigenic component of gram positive bacteria, has been found in endothelial cells [68], while Helicobacter and lipopolysaccharide have also been described in PBC livers [69, 70]. ET2 may in turn stimulate Kupffer cells to produce pro-inflammatory cytokines, such as IL-1 and IL-6 from mouse peritoneal macrophages (but not TNFα or NO in this particular model) [71]. ET2 is also a potent macrophage chemoattractant [72] via the ETB receptor. ET2 shares the similar peptide sequence with CXC chemokines.

In accordance with this hypothesis, macrophages constitute 30% of the cellular infiltrate on portal areas and around damaged bile ducts [73]. Activated macrophages have also been observed by electron microscopy near epithelial cells of the bile ductules and seem to develop into epitheliod cells [74]. Epitheliod granulomata of PBC patients contain most MCP2 and MCP3 positive cells at their edge and more than 60% of them co-express CD68, indicating that they are derived from macrophages [75].

In stages 3 and 4, PBC Kuppfer cells and myofibroblasts are increased in periportal and periseptal areas, possibly indicating that Kupffer cells interact with stellate cells and lead to fibrosis [76], thus forming the connecting element to the development of cirrhosis.

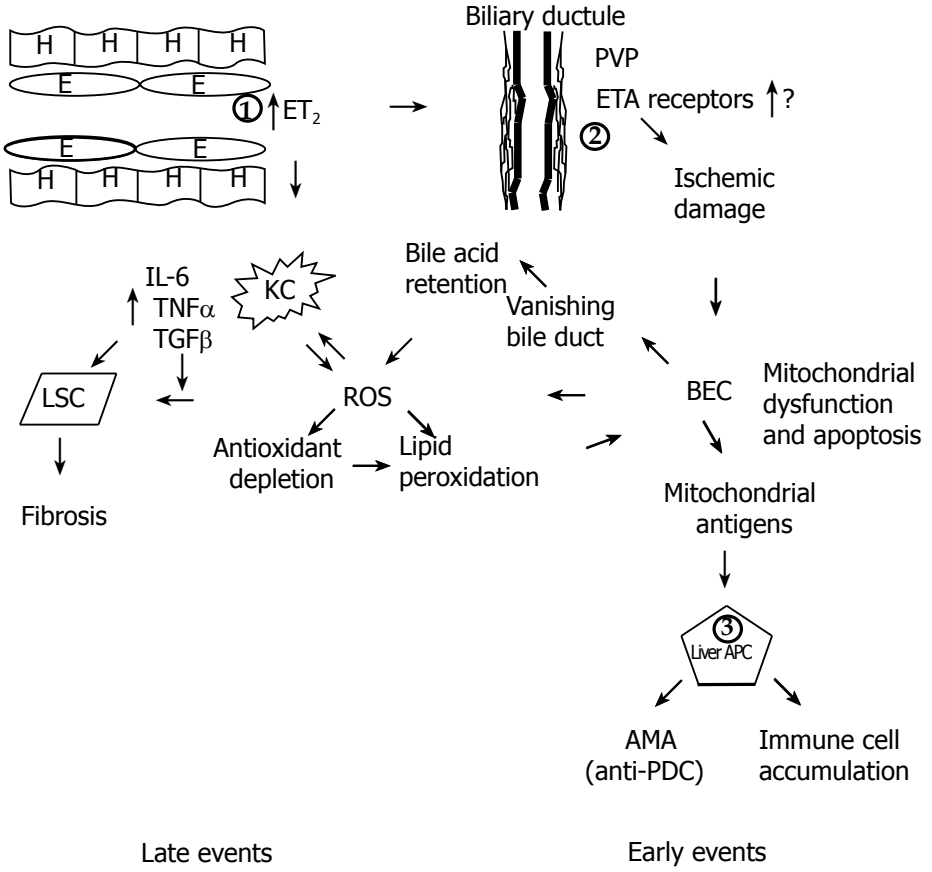

Figure 1 Figure 1 Occurrence of late and early events in the unifying model. The fundamental early defect in PBC is the overproduction of ET2 by endothelial cells (1) possibly driven by virus [95-98] or other microbial pathogens in genetically predisposed individuals. ET2 is a chemoattractant for Kupffer cells and also causes contraction of stellate cells leading to early portal hypertension. ET2 leads to ischemic damage of BEC (2) through constriction of the PVP with resultant BEC mitochondrial dysfunction and membrane disruption by ROS generation and eventually apoptosis leading to the vanishing bile duct syndrome. Mitochondrial antigens possibly generated through caspase cleavage, are presented by hyper-responsive liver dendritic cells (APC) (3) and lead to immune cell accumulation and AMA production. The second fundamental defect occurring later in the disease process is the production of ROS. Together with ingestion of BEC apoptotic bodies, ROS drives the accumulated Kupffer cells to produce more ROS and pro-inflammatory cytokines and TGFβ, which in turn leads to fibrosis. ROS is also produced after development of the vanishing bile duct syndrome as a result of bile acid retention. Finally through antioxidant depletion or through an insufficient increase of antioxidants ROS leads to lipid peroxidation and further BEC apoptosis and mitochondrial dysfunction. Genetically determined control may be exercised at levels 1,2 and 3.

Endothelins also cause contraction of stellate cells [77] and possibly help their differentiation to myofibroblasts. This contradiction may play an important role in the development of early portal hypertension in PBC as has been suggested in rats with biliary cirrhosis [61, 76-78].

Intrahepatic bile ducts receive their blood supply from a periductal network of minute vessels, known as the peribiliary vascular plexus (PVP). This plexus originates from hepatic artery branches accompanying its intrahepatic bile duct [79] and drains mostly into the sinusoids [80]. The peribiliary space also contains dendritic cells and stellate cells. Insufficient perfusion of this system can cause profound bile duct damage [81]. ETs and NO seem to be the principal molecules that regulate circulation of the PVP [82]. It is suggested in our model that patients with PBC have damage to the biliary endothelium as an initiative event caused by ischaemia due to ET-2 driven vasoconstriction. There is direct evidence that the peribiliary capillary plexus is indeed damaged in PBC. It was reported that the peribiliary plexus is significantly reduced in PBC (and interestingly also in auto-immune hepatitis), while there is proliferation of the plexus in other liver diseases [83]. There are also other reports indicating vascular impairment in PBC [61,78]. The increased circulating ET-2, observed both in early and late stage PBC seems to be specific for this disease, since it has not been found in the disease control groups of cirrhotics and patients with chronic viral liver disease.

Biliary ischemia might then lead to apoptosis of biliary epithelial cells, which is indeed a mechanism proposed for biliary epithelial destruction in PBC [84, 85]. Moreover biliary epithelial cells (BEC) undergoing apoptosis release the pyruvate dehydrogenase complex (PDC) from mitochondria into the cytoplasm as early as 6 hours after induction of apoptosis and auto-reactive epitopes are present in BEC, while other cells efficiently delete cytoplasmic PDC by glutathione [43,86]. Such a mechanism may also explain the similarities between PBC and graft *vs* host disease (GVHD) [87-90]. GVHD is associated with endothelial cell injury [91] and IL-1 has been implicated in its pathogenesis [92]. More importantly, in GVHD after small bowel transplantation, ET1 levels are increased before the induction of GVHD and have been histochemically shown to be increased in endothelial and epithelial cells some days before GVHD, implicating a pathogenetic significance [93]. Immunoreactive epitopes, self-PDC generated during apoptosis, possibly through the action of caspase 3 [43] are taken up by either the peribiliary dendritic cells or by BEC expressing MHC II (this could be either genetically determined or alternatively be caused by pro-inflammatory cytokines [94]). In the first instance this leads to the production of auto-antibodies and possible the determinant density model as elegantly described by Jones [1].

There are many questions that have yet to be answered regarding the above suggested model of liver injury in PBC. The most important issue is that the increased concentration of ET-2 has been found in

systemic circulation. This means that the vasoconstriction and the consequent ischemic injury ought to happen in many organs apart from liver and PVP. A possible explanation for this selectivity is an increased expression of ET receptors in the PVP of PBC patients but this suggestion needs to be further studied.

The suggested model is diagrammatically outlined in Figure 1. However, the proposed model has the following advantages. It implicates both innate and adaptive immunity. The former is the initiating event while the latter is the element that causes perpetuation of the disease even after disappearance of the initial event (if this is environmental infections). It explains the role of infective agents and the similarity of PBC with graft vs host disease. Interaction of endothelial and Kupffer cells with stellate cells explains the progress to fibrosis and cirrhosis. It predicts that most infiltrating cells should be CD4 helper T cells participating in B cell differentiation as it is indeed the case [31-35] but the role of CD8 is limited [37,38]. Since AMA production is a secondary phenomenon rather than pathogenetically related to liver damage, the presence of AMA negative PBC is also explained. Ursodeoxycholate (UDCA) may act mostly as an R0S scavenger preventing the mitochondrial oxidative stress. Most importantly, it offers 3 levels for a genetically determined control, namely the level of endothelial cells (and possibly Kupffer cells), the level of perivascular plexus and ET receptor expression, and the level of peribiliary dendritic cells that might be genetically hyper-responsive. All of them may well be estrogen dependent, thus explaining the extreme female prevalence of the disease, but this requires further research.

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REVIEW

Eosinophilic esophagitis: A newly established cause of dysphagia

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Abstract

Eosinophilic esophagitis has rapidly become a recognized entity causing dysphagia in young adults. This review summarizes the current knowledge of eosinophilic esophagitis including the epidemiology, clinical presentation, diagnostic criteria, pathophysiology, treatment, and prognosis. An extensive search of PubMed/Medline (1966-December 2005) for available English literature in humans for eosinophilic esophagitis was completed. Appropriate articles listed in the bibliographies were also attained. The estimated incidence is $43/10^5$ in children and $2.5/10^5$ in adults. Clinically, patients have a long history of intermittent solid food dysphagia or food impaction. Some have a history of atopy. Subtle endoscopic features may be easily overlooked, including a "feline" or corrugated esophagus with fine rings, a diffusely narrowed esophagus that may have proximal strictures, the presence of linear furrows, adherent white plaques, or a friable (crepe paper) mucosa, prone to tearing with minimal contact. Although no pathologic consensus has been established, a histologic diagnosis is critical. The accepted criteria are a dense eosinophilic infiltrate (>20/high power field) within the superficial esophageal mucosa. In contrast, the esophagitis associated with acid reflux disease can also possess eosinophils but they are fewer in number. Once the diagnosis is established, treatment options may include specific food avoidance, topical corticosteroids, systemic corticosteroids, leukotriene inhibitors, or biologic treatment. The long-term prognosis of EE is uncertain; however available data suggests a benign, albeit inconvenient, course. With increasing recognition, this entity is taking its place as an established cause of solid food dysphagia.

Key words: Eosinophilic esophagitis; Allergy; Dysphagia

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INTRODUCTION

The esophagus normally is rather devoid of eosinophils. Not merely a simple conduit for swallowing food and liquids, the esophagus is being recognized as an immunologically active organ that can respond to a variety of stimuli like gastric acid and allergens by recruiting eosinophils and eliciting an inflammatory response. Eosinophils, for example, commonly infiltrate the lower esophagus in conjunction with gastroesophageal reflux disease (GERD)[1] A relatively new entity, eosinophilic esophagitis, with more extensive eosinophilic infiltration, particularly in the proximal esophagus[2] possesses clinical features that differ from GERD[3]. This entity is becoming increasingly recognized as a cause of dysphagia, often with a history of food impaction[4]. Since its original description in 1978[5], EE has exploded onto the clinical scene, becoming a recognized cause of solid food dysphagia, first identified in the pediatric and now the adult population[6-8]. Eosinophilic esophagitis is a disorder in which eosinophils infiltrate the superficial mucosa of the esophagus. Previously thought to be a rarity, case reports of eosinophilic esophagitis are rapidly accumulating in the literature[3,7]. Clinical presentation and the endoscopic-[7] and histological[7] features have become more firmly established, although a consensus is still lacking for an absolutely clear-cut diagnosis. Eosinophilic esophagitis has been associated with food allergies and atopic conditions such as asthma and atopic dermatitis[6,8-10].

Eosinophilic esophagitis is better known in the pediatric population through several published studies and reviews[11-14]. Infiltration of eosinophils into the esophagus may result from conditions such as food allergy, infection, gastroesophageal reflux disease (GERD), or systemic eosinophilic conditions. The mechanism of dysphagia from eosinophilic esophagitis has yet to be defined.

This review summarizes the epidemiology, clinical presentation, possible pathophysiological mechanism, diagnosis, treatment, and prognosis for eosinophilic esophagitis, primarily focusing on adults with this condition.

Table 1 Clinical features of eosinophilic esophagitis

	Adult	Pediatric
Common	Dysphagia Food impaction/foreign body Esophageal stricture Nausea/vomitting/regurgitation Heartburn Food allergy	Abdominal pain Failure to thrive Nausea/vomitting Dysphagia Food allergy Heartburn
Uncommon	Hematemesis Globus Waterbrash Weight loss Chest pain Abdominal pain	Food impaction
Associated Conditions	History of atopy Asthma Allergic rhinitis	Asthma Allergic rhinitis Eczema Atopic dermatitis Strong family history of atopy

EPIDEMIOLOGY

Epidemiological studies on eosinophilic esophagitis are lacking, likely from inadequate recognition and a paucity of established diagnostic criteria. Most publications are case reports or case series. Information is more widely available for the pediatric population compared to adults. This may be due to increased aggressiveness in investigating children with GI symptoms, or practice habits of pediatric gastroenterologists performing random biopsies in all cases. Fox *et al*[15] estimated that 6.8% of children with esophagitis had eosinophlic esophagitis, while Liacouras *et al*[16] indicated 3.4% of such children experienced reflux symptoms. A previous estimate of frequency was 1 per 100 000[17]. A recent population based study by Noel *et al*[13] in Ohio based on 103 children suggested a much higher figure: an annual incidence of 1 per 10 000 and a prevalence of 4.296 per 10,000 children, which rose over the period of the study - from 2 000 to 2003. Whether this represented a true increase in the entity or bias from improved detection is unknown. A strong familial pattern was evident. In Italy the prevalence was reported to be 3.5%[18]. A worldwide pediatric registry has been established[19].

Population-based data is lacking in the adults. Croese *et al*[20] identified eosinophilic esophagitis in 19 adult patients from a population of 198 000 over a 21 months period. The study, however, used 30 eosinophils per high power field as its criteria for diagnosis, a value higher than in most studies (usually >20/high power field). Therefore the incidence may be underestimated. The study also included both pediatric and adult populations with an age range of 14-77. Nevertheless, eosinophilic esophagitis appears to be an increasingly recognized entity with an accelerating frequency[13,21,22].

DIAGNOSIS

The diagnosis of eosinophilic esophagitis is based on clinical presentation, endoscopic or radiographic features, and histopathological criteria.

Clinical features

Clinical features (Table 1) of eosinophilic esophagitis have been previously well defined[13,20,22,23]. There is a male predilection and a wide range of ages from pediatric to adult populations. Mean age in children ranges from 7-10 years, and 30-40 years in adults. Dysphagia is the most common symptom in adults and is usually longstanding. Food impaction, reflux symptoms, vomiting or regurgitation, and food allergy are also common. Abdominal pain (30%), vomiting (30%) and failure to thrive (20%) are more common in the pediatric population compared to only 3% adults with abdominal pain, however there may be a selection bias based on more aggressive evaluation of these symptoms in children compared to adults. The majority of the pediatric population will have a history of atopic conditions, such as asthma, allergic rhinitis, eczema, or atopic dermatitis[24]. Noel *et al*[13] found 57.4% of children with eosinophilic esophagitis had a history of rhinoconjunctivitis, 36.8% wheezing, 46% possible food allergy, and 73.5% a family history of atopy. Adults also may have a history of atopy, but this is not as prevalent as in children. Croese *et al*[20] found that 46% of adults with eosinophilic esophagitis had a history of atopy, and only 25% food allergy. No relation has been found to connective tissue diseases such as scleroderma, rheumatoid arthritis, or lupus. Uncommon symptoms include hematemesis, globus, and waterbrash.

Laboratory features have not been extensively reported in eosinophilic esophagitis, therefore sensitivity and specificity of laboratory tests are unknown. Peripheral blood eosinophilia range from 5%-50% in the adult population with eosinophilic esophagitis. Increased serum IgE, positive skin prick or radioallergosorbent test (RAST) may be found in 40%-73% of patients[6,20]. In a study of 26 children, 19 tested positive for skin prick testing, and 21/26 had positive patch testing[25]. Skin testing may therefore help to identify causative food agents. These cases of rather overt immediate hypersensitivity are often not apparent in the adult patient. Indeed, food allergies in childhood may not persist to adulthood. Limited studies are available on the use of these laboratory values for the diagnosis of eosinophilic esophagitis.

Radiological features

The most common diagnostic imaging test that to date has detected eosinophilic esophagitis is a barium study[26,27]. Zimmerman *et al* retrospectively assessed 14 patients with confirmed eosinophilic esophagitis and found 10 with strictures (mean length 5.1 cm), of whom 7 had multiple fixed ring-like indentations. Four patients had esophagitis, 10 hiatus hernia, and 9 with evidence of reflux[27].

Endoscopic features

The "feline esophagus", also known as the "corrugated esophagus", "ringed esophagus", or "concentric mucosal rings", is the classic endoscopic description of eosinophilic esophagitis (Table 2, Figure 1)[7,20]. A small caliber esophagus with a narrow fixed internal diameter, with or without a proximal esophageal stenosis, may also be the major feature[28,29]. Adherent white exudates, vesicles, or papules along with loss of vascular pattern may indicate focal areas

Table 2 Endoscopic features of eosinophilic esophagitis

Endoscopic feature	Description
Feline esophagus (corrugated, ringed esophagus)	Multiple concentric rings, may be fine in nature, web-like or thickened
Small calibre esophagus	Narrow, fixed internal diameter Featureless, unchanging column Poor expansion on air insufflation Proximal and/or distal stenosis
Adherent white papules	White exudates 1-2 mm in diameter which do not wash off (similar to candidiasis) Speckled patches Vesicles Loss of vascular pattern
Esophageal furrows	Vertical esophageal lines
Crêpe paper mucosa	Fragile esophageal mucosa Delicate, inelastic Mucosal abrasions or tear with minimal contact

Table 3 Histopathology of eosinophilic esophagitis

	GERD	Eosinophilic esophagitis
Eosinophilic infiltration (squamous epithelium)	<10/hpf	>20/HPF
Other features	Esophagitis (usually distal)	Esophagitis (proximal and/or distal, may be patchy or segmental)
	Intestinal metaplasia	Basal zone hyperplasia
		Increased papillary size
		Superficial eosinophilic layering or aggregates
		Microabscesses

GERD: Gastroesophageal reflux disease; HPF: high power field.

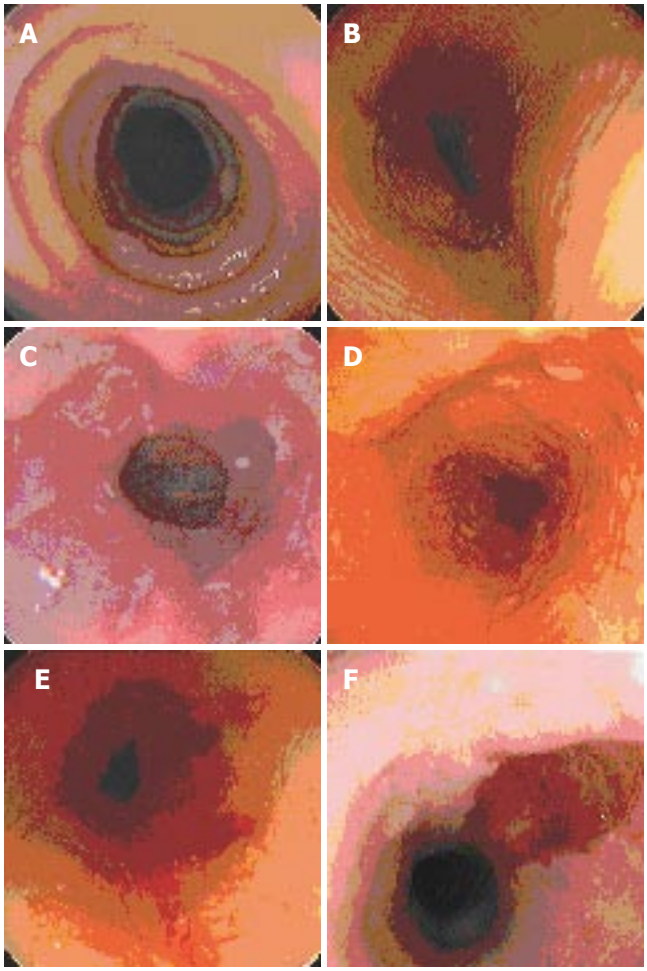

Figure 1 Classic endoscopic features of EE. **A**: Coarse, corrugated, narrow esophagus with papules; **B**: Fine feline esophagus; **C**: Distal esophageal stricture; **D**: Diffusely narrow esophagus with adherent white papule; **E**: Friable, crêpe paper mucosa with linear furrows; **F**: Large esophageal tear after biopsy.

of eosinophilic infiltration[30,31]. Vertical esophageal lines also may indicate eosinophilic esophagitis[32]. Finally, the esophageal mucosa may be fragile, or the so called "crêpe paper mucosa"[33]. This would explain the frequency of esophageal tears following dilation when treating the dysphagia associated with an apparently narrowed esophagus or its ringed structure (appearing like stricture). Thus the fragile esophagus is also characteristic. Endoscopic ultrasound, when performed, will show circumferential but asymmetric thickening of the muscularis propria[34]. The most common endoscopic findings in one relatively large series[35] were, in order of frequency: mucosal rings (81%), furrows (74%), strictures (31%), exudates (15%), small caliber (10%) and edema (8%). The endoscopic appearance is helpful but not diagnostic without a confirmatory biopsy. All patients with endoscopic features of eosinophlic esophagitis should have distal and proximal eosophageal biopsies to confirm eosinophilic esophagitis. Furthermore, this should be assessed prior to mechanical dilatation of strictures, as medical treatment for eosinophilic esophagitis should be the initial treatment. There have been no studies assessing the histopathological diagnosis of eosinophilic esophagitis in those with dysphagia and normal endoscopy. Therefore, it is unclear whether all these patients should have the proximal esophagus biopsied.

Histopathology

The diagnosis of eosinophilic esophagitis is dependent on eosinophilic infiltration of the squamous epithelium (Table 3). Although there is no consensus statement, most studies agree that >20 eosinophils per high power field (HPF) are diagnostic of eosinophilic esophagitis[6,36,37]. GERD can increase eosinophilic infiltration in the distal esophagus and therefore, mid or upper esophageal biopsies with increased eosinophils is more specific for eosinophilic esophagitis. Nevertheless, increased tissue eosinophils associated with GERD occur at a lower density <10/HPF[36,37] (Table 4).

Other features that are helpful but not essential for the diagnosis include basal zone hyperplasia, increased papillary size, and superficial layering of eosinophils with aggregates or microabscesses (aggregate of 4 or more contiguous eosinophils).

PATHOPHYSIOLOGY

Eosinophils originate in the bone marrow. When mature, only a small number circulate in the peripheral blood; rather they are predominantly tissue-dwelling cells. In health, other than hematopoietic sites, eosinophils only reside in the lamina propria of the gastrointestinal tract, the exception being the esophagus. Resident in the

Table 4 Differential diagnosis [40]

Primary	Idiopathic eosinophilic esophagitis Familial eosinophilic esophagitis Atopic esophagitis
Secondary: Eosinophilic related	Eosinophilic gastroenteritis Hypereosinophilic syndromes
Secondary: Non – eosinophilic related	GERD Recurrent vomitting Infection (helminth, parasitic, fungal) Esophageal GI stromal tumor Myeloproliferative disorders Carcinomatosis Allergic vasculitis Scleroderma Drugs/Iatrogenic

Table 5 Treatment regimens for eosinophilic esophagitis

Treatment option	Protocol
Elimination Diet	Avoidance of allergen depending on results of food allergy testing Oligoantigenic diet: Eliminate large number of suspected foods and allow limited nutritionally balanced diet Elemental diet: Various formulas such as Neocate (free amino acids, corn syrup solids, medium chain triglycerides)
Topical corticosteroids: Mayo Clinic protocol	Fluticasone 220 μg puffer 4 puffs BID x 6 wk, swallowed, no spacer Rinse mouth with water and spit out No food or drink for 3 h after dose
Systemic (oral) corticosteroids	Methylprednisolone 1.5 mg/kg per day (or equivalent dose prednisone) Divide into bid dosing for 4 wk then taper over 6 wk
Montelukast	Initial dose: 10 mg *po* daily Titration: Dose up to 100 mg/d depending on symptoms and tolerance Maintenance: Once symptoms relieved titrate down to minimal dose to maintain remission (usually 20 - 40 mg/d)
Mepolizumab	10 mg/kg *iv* infusion q 4 wk x 3 doses

BID: twice daily; PO: oral; IV: intravenous.

gastrointestinal tract, eosinophils normally do not evoke either an inflammatory reaction or tissue damage[38]. Under inflammatory conditions, eosinophils can infiltrate several organs (e.g., lung, esophagus and GI tract, and skin), playing a major role in causing tissue damage and organ dysfunction, and being mediators of allergic responses such as atopic dermatitis, allergic rhinitis, and asthma. Eosinophilic esophagitis characteristically has a dense eosinophilic infiltrate confined to esophageal tissues[21,36]. Activation of eosinophils results in degranulation, upregulated cytokine production, and IgE production. Recruitment and activation is regulated by cytokines including interleukin 5 (IL-5), eotaxin, interleukin 13 (IL-13), and interleukin 4 (IL-4)[38,39,40].

IL-5 is a critical cytokine for the differentiation and activation of eosinophils[38,41]. In eosinophilic esophagitis, a Th2 allergic response and production of IL-5 is key in recruitment of eosinophils to the esophagus. Mice devoid of IL-5 or lacking the receptor for IL-5 have a significant reduction in GI eosinophils. Overexpression of IL-5 can promote eosinophilic accumulation[38]. Eotaxin is constitutively expressed in the GI tract and has a critical role in eosinophilic recruitment[38]. Transgenic IL-5 mice deficient in eotaxin fail to recruit eosinophils to the GI tract. It seems to be more important for chemotaxis to the stomach and intestine[40,42]. Fujiwara, however, showed significant staining of eosinophils with anti-eotaxin-antibodies in patients with EE[43].

IL-13 is a profibrotic cytokine and its production has been demonstrated in eosinophils. Likely Th2 mediated, IL-13 contributes to an inflammatory response and bronchial hyperreactivity[10,44]. Intratracheal IL-13 was shown to induce eosinophilic esophagitis, linking pulmonary to esophageal eosinophilic inflammation[45]. It appears that IL-13 is a key mediator of eosinophilic inflammatory pathways and in the recruitment of eosinophils to the esophagus[10,45]. IL-13 may be a serologic indicator of systemic inflammation[46]. IL-13 induction of eosinophilic esophagitis seems to be dependent on IL-5, eotaxin, and STAT-6[45]. Interestingly, mepolizumab, an anti-IL-5 antibody, was shown to be beneficial for symptomatic and histologic improvement in eosinophilic esophagitis[47]. It is possible that the interaction of IL-13, IL-5 and eotaxin may be a component to the development of eosinophilic esophagitis. IL4 and IL-13 share a signal transduction pathway involving IL-4 receptor α chain and STAT-6[43]. IL-4 has been implicated in eosinophilic accumulation, regulating trafficking, and promoting adhesion to endothelial surfaces. Increased IL-4 secreting T cells in esophageal lesions were evident in one trial of patients with secondary eosinophilic esophagitis[48], but do not induce eosinophilic infiltration into the murine esophagus[43].

The net result of such chronic inflammation is irreversible structural change with loss of mucosal elasticity and the development of fibrosis in the subepithelial layers[21].

TREATMENT

The majority of reports on eosinophilic esophagitis are case reports or series. Therefore, randomized controlled trials (RCTs) for eosinophilic esophagitis treatment are not available. Indeed, a recent Cochrane review did not yield any RCTs, nor were the authors able to make any conclusions on benefits and harms of treatment regimens[49]. Treatment falls into two categories: (1) avoidance/removal of stimulation and (2) immune modulation. The majority of studies have been published in pediatric literature (Table 5). Avoidance of stimulation involves dietary changes with the elimination of foods or an elemental diet. Given that skin testing may help identify causative foods[25,27], this may help in avoidance of the specific culprit in some cases. Skin prick and skin patch testing may be more effective than skin prick testing alone. As shown by Spergel *et al*[25] in 26 children with documented eosinophilic esophagitis, 68 foods were identified in 19/26 by skin prick testing, and 67 foods in 21/26 by skin patch testing, for an average

of 2.7 foods per patient. With specific food avoidance, 18 had complete resolution and 6 partial improvement. Kelly *et al*[50] used an elemental diet in 10 children with eosinophilic esophagitis, and showed partial or complete resolution of symptoms in all 10. Markowitz *et al*[51] conducted a study in 346 children with chronic GERD symptoms of which 51 were eventually diagnosed with eosinophilic esophagitis. They were then given an elemental formula (Neocate 1+, SHS Northa America, Gaithersburg, MD) consisting of free amino acids, corn syrup solids, and medium chain triglyceride oil. Forty-eight patients were fed via a nasogastric tube, 49/51 patients improved symptomatically and there was a significant decrease in the number of eosinophils in the distal esophagus. Average time to improvement was 8.5 d. Unknown is if any of these measures of food avoidance or elemental diets are effective in adults.

Topical steroid therapy has been shown to be helpful in a number of uncontrolled case series reports for both the pediatric[52,53] and adult populations[54]. Arora *et al* treated 21 adult patients with eosinophilic esophagitis (diagnosed via solid food dysphagia, ringed esophagus, and eosinophils >20/hpf in mid to distal esophagus) with a 6 wk regimen of fluticasone 220 μg 4 puffs swallowed twice daily. All patients had complete symptomatic relief for at least 4 mo. The only side effect was dry mouth, with no oral candidiasis reported. Three out of 21 patients had relapse at 4 months and 50%-60% of patients had recurrence of symptoms at 1 year[6,54]. Systemic steroid therapy was first reported by Liacouras *et al* in the pediatric population[16]. Of 1809 patients with reflux, 20 had documented eosinophilic esophagitis and were treated with 1.5 mg/kg oral methylprednisolone divided twice daily for 4 wk. Steroids and anti-reflux medications, such as proton pump inhibitors, were then tapered and withdrawn after 6 wk. Thirteen out of 20 patients had a complete response and 6/20 marked clinical improvement (total 19/20 responders). Average time to improvement was 8 d. All had histologic evidence of improvement and a significant decrease in peripheral eosinophil counts and quantitative IgE levels. At 1-year follow-up, 10/20 were asymptomatic and 9/20 relapsed. Relapsers were treated with dietary changes, of which two required a second course of oral steroids. A randomized controlled trial comparing oral to inhaled corticosteroids is ongoing.

Leukotrienes promote eosinophilic trafficking, smooth muscle constriction, and mucous hypersecretion. Eosinophils generate large quantities of leukotriene C4, which is then metabolized to leukotriene D4 and E4 (LTD4 and LTE4 respectively). Montelukast is a selective inhibitor of the LTD4 receptor. Attwood *et al* reported 12 adult patients with dysphagia secondary to eosinophilic esophagitis and investigated the use of montelukast in 8/12 [55]. Patients were given an initial dose of montelukast 10 mg orally once daily and titrated up to a total of 100 mg daily. Once symptoms were relieved, dose was reduced to a "maintenance level" (20-40 mg/d). All patients were previously treated with proton pump inhibitors and 2 previously responded to corticosteroid treatment. All patients had symptomatic improvement, with only 2 having residual discomfort. Patients have been treated for a median of 14 months with no relapse. Six out of 8 experienced recurrence of symptoms within 3 wk of dose reduction or cessation. Important side effects were nausea and myalgias. Treatment did not change the density of eosinophils on repeat biopsy.

The central role of IL-5 in eosinophilic regulation and activation makes it a viable target for therapy. Mepolizumab is a humanized anti-IL-5 monoclonal antibody shown to be safe and effective in reducing sputum eosinophils in asthma but ineffective in outcome measures[56]. Garrett *et al*[47] performed an open label pilot study on 4 patients with hypereosinophilic syndromes, of which 3 had idiopathic hypereosinophilic syndrome and only 1 patient had eosinophilic esophagitis. This patient had dysphagia, esophageal narrowing on endoscopy with marked eosinophilia on biopsy, and was unresponsive to dietary elimination, topical, and oral corticosteroid treatment. Three doses of mepolizumab (10 mg/kg intravenous) infused at 4 wk intervals were given and patients followed for 18 wk after first infusion. Remarkable symptomatic improvement was achieved. Endoscopic and histologic improvement was seen at 4 wk after the last infusion. Peripheral eosinophils were reduced immediately after the first infusion and continued to the end of follow up. No serious adverse events were noted. No larger trials have been published. Other medications successfully used in eosinophilic gastroenteritis such as cromolyn and ketotifen (mast cell stabilizing medications), and suplatast tosilate (selective Th2 IL-4 and IL-5 inhibitor) have not been studied in eosinophilic esophagitis[19].

PROGNOSIS

Esposito *et al*[18] followed 7 children with eosinophilic esophagitis for 4 years, ages ranging from 6 months to 14 years old. All were treated with inhaled fluticasone. Two children experienced relapse at 1 year and 4 years post treatment, respectively, which improved with a second course of inhaled corticosteroid. Compliance was low in 2 patients and both had poor clinical and histologic response. Repeat treatment with appropriate dosing cured their symptoms. All children had normal growth after treatment. Interestingly, density of eosinophilic infiltrate was inversely proportional to age, and progressively reduced with time. This may explain the higher incidence in children compared to adults. Liacouras *et al*[16] found a 50% one year relapse rate after a course of oral steroids. Finally, Orenstein *et al*[14] found that 1/3 of patients were asymptomatic without any therapy.

Straumann *et al*[21] documented the natural history of eosinophilic esophagitis in 30 adult patients. Mean age was 40.6 and mean follow up time was 7.2 years. None were treated with dietary changes or medical therapy. Only those with severe and frequent attacks were treated with dilatation. No patients died and all were in "good health" with maintenance of body weight. Twenty-nine of 30 (96.7%) patients had dysphagia throughout follow up: 7 experienced increasing dysphagia, 11 persistent but stable dysphagia, 11 decreasing dysphagia, and 1 complete resolution. Eleven out of 30 required dilatation, of which 10 had reduction or cure of dysphagia. In terms of the impact of

dysphagia on quality of life, 1/30 reported a significant negative impact on socioprofessional activities, 15/30 minor, and 14/30 reported no significant impact. No increased risk of malignancy was found and no eosinophilic gastroenteritis was documented. It appears that in the adult population disease tends to be stable with no significant effect on morbidity or mortality, at least for up to 11 years' follow up. Whether a persistent inflammatory state will affect motility, mechanical obstruction, inflammatory bowel disease, malignancy, or mortality in the long term has yet to be seen.

SHORTFALLS

A lack of consensus for diagnosis hinders research progress in eosinophilic esophagitis. Case descriptions to date have used variable cutoffs for eosinophilic infiltration ranging from >15/HPF to >30/HPF. This not only affects the epidemiological data for incidence and prevalence, but also affects the inclusion/exclusion into trials and histological response to therapy. Furthermore, no distinction is made between proximal and distal esophageal biopsies, which might influence the pathologist into the diagnosis of esophagitis secondary to reflux rather than idiopathic eosinophilic esophagitis. Biopies are essential for the accurate diagnosis of eosinophilic esophagitis and are best taken from the proximal esophagus to better distinguish this entity from reflux esophagitis, even though the latter has a less dense eosinophilic infiltrate.

No objective criteria have been developed to assess the response to treatment. All studies to date have employed subjective improvement in symptoms, which is prone to bias. No randomized controlled trials to date have validated the efficacy of any treatment regimen. Elimination diets are inconvenient and result in low compliance. Topical steroid treatment seems safe[54] and may be the most convenient and effective therapy in adults, though technique may be an issue. Oral corticosteroid has its myriad of side effects and complications. Montelukast may be effective but would be long-term treatment given its relapse rates off treatment. Given its benign natural history in which the majority of patients have minor or no impact on quality of life, these therapies should be validated with vigorous clinical trials that include an analysis of cost effectiveness.

Finally, what should be done with the non-responsive patient? Compliance should definitely be confirmed. Repeat treatments may be beneficial. Combination therapy has yet to be explored. New biologic agents may be beneficial, but efficacy needs to be confirmed, and cost will be a limiting factor.

CONCLUSION

Since its original description, most publications have emphasized the clinical and histopathological presentation of eosinophilic esophagitis. The epidemiology is being better understood as clinicians are recognizing this unique disease entity. Diagnostic criteria are evolving which will improve the quality of future research. A consensus on the diagnosis of eosinophilic esophagitis is urgently needed. Despite a plethora of case reports, case series, case cohorts and reviews, randomized placebo-controlled trials are needed to confirm the efficacy of treatment regimens. In adult patients, topical corticosteroid appears to be the most convenient and efficacious treatment. As the pathophysiology of eosinophilic recruitment and activation in the esophagus is further elucidated, future treatment targets are possible. The long term natural history and response to treatment is awaited. Eosinophilic esophagitis may be a relatively new entity, undoubtedly overlooked in the past but this disease is here to stay. With better recognition, it has moved into the forefront of esophageal diseases.

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Helicobacter infection in hepatocellular carcinoma tissue

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Abstract

AIM: To investigate whether *Helicobacter* species (*Helicobacter* spp.) could be detected in hepatocellular carcinoma (HCC) tissue.

METHODS: Liver samples from 28 patients with hepatocellular carcinoma (HCC) diagnosed by histopathology were studied. Twenty-two patients with other liver diseases (5 with liver trauma, 7 with cavernous liver hemangioma, 6 with liver cyst and 4 with hepatolithiasis), 25 patients with gastric cancer, 15 with colonic cancer and 15 with myoma of uterus served as controls. Two pieces of biopsy were obtained from each patient. One was cultured for *Helicobacter* spp. and extraction of DNA, the other was prepared for scanning electron microscopy (SEM) and *in situ* hybridization. The samples were cultured on Columbia agar plates with microaerobic techniques. *Helicobacter* spp. in biopsy from the studied subjects was detected by polymerase chain reaction (PCR) with *Helicobacter* spp. 16S rRNA primers. Amplified products were identified by Southern hybridization and sequenced further. Besides, other genes (*vacA, cagA*) specific for *Helicobacter pylori* (*H pylori*) were also detected by PCR. *Helicobacter* spp. in biopsies was observed by SEM. Transmission electron microscopy (TEM) was performed to identify the cultured positive *Helicobacter* spp. The presence of *Helicobacter* spp. was detected by *in situ* hybridization to confirm the type of *Helicobacter*.

RESULTS: The positive rate of *Helicobacter* cultured in HCC and gastric cancer tissue was 10.7% (3/28) and 24%(6/25), respectively. *Helicobacter* microorganisms were identified further by typical appearance on Gram staining, positive urease test and characteristic colony morphology on TEM. The bacterium was observed in adjacent hepatocytes of the two HCC samples by SEM. The number of cocci was greater than that of bacilli. The bacterium was also found in four gastric cancer samples. PCR showed that the positive rate of HCC and gastric cancer samples was 60.7% and 72% respectively, while the controls were negative ($P < 0.01$). The PCR-amplified products were identified by Southern hybridization and sequenced. The homology to 16S rRNA of *H pylori* was 97.80%. The samples were verified by *in situ* hybridization for *Helicobacter* spp. 16S rRNA–mRNA and proved to be *H pylori* positive. There was no statistical significance between HCC and gastric cancer ($P > 0.05$), but the positive rate of HCC and controls had statistical significance ($P < 0.01$). Only 3 HCC samples and 2 gastric cancer samples of the cagA genes were detected. None of the samples reacted with primers for vacA in the two groups. As for the genotype of *H pylori*, type II had preference over type I.

CONCLUSION: *Helicobacter* infection exists in liver tissues of HCC patients. *Helicobacter* spp. infection is related with HCC, which needs further research.

Key words: Hepatocellular carcinoma; *Helicobacter* infection

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INTRODUCTION

The profound impact of HCC on human health is known worldwide[1]. In China, the death rate ranks the third in malignant tumors. Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and aflatoxins are the main causes of HCC[2]. The real risk factors for HCC may be far more than the known causes. A new infectious agent, *Helicobacter hepaticus* (*H hepaticus*) causing chronic active hepatitis and associated liver tumors has been described by Ward *et al*[3]. Recently, other *Helicobacter* species (*Helicobacter* spp.), including *H pylori* and other bacteria associated with the pathogenesis of gastric[4-7] and extradigestive manifestations[8,9], have been detected in the liver of patients suffering from cholestatic diseases and HCC arising from non-cirrhotic liver[10,11]. We have previously reported a high frequency of *Helicobacter* spp.

in the liver of patients with HCC[12]. *Helicobacter* spp. DNAs have been detected in paraffin-embedded tissue sections of HCC by PCR[13]. Whether *Helicobacter* spp. promotes liver tumors or acts as a cofactor in the process of carcinogenesis in humans with hepatitis virus needs further research. This study was to determine whether *Helicobacter* spp. could be detected in HCC tissue and to investigate the potential significance of *Helicobacter* in HCC carcinogenesis.

MATERIALS AND METHODS

Samples

All samples were immediately frozen in liquid nitrogen and stored at -80℃ before testing. Liver samples from 28 patients with HCC (25 males, 3 females, mean age 54 years) were studied. Twenty patients (18 males, 4 females, mean age 48 years) with other liver diseases (5 with liver trauma, 7 with cavernous liver hemangioma, 6 with liver cyst and 4 with hepatolithiasis), 25 with gastric cancer (20 males, 5 females, mean age 61 years), 15 with colonic carcinoma (9 males, 6 females, mean age 55 years) and 15 with myoma of uterus (mean age 49 years) served as controls. Two pieces of biopsy were obtained from each patient, one was cultured for *Helicobacter* spp. and extraction of DNA, the other was prepared for scanning electron microscopy (SEM) and *in situ* hybridization.

Germ culture

To culture *Helicobacter* spp., the diluted homogenates of biopsy specimens were smeared on the surface of Columbia agar plates (Oxoid Company, France) supplemented with 7% sheep erythrocytes, 6 μg/mL vancomycin, 2 μg/mL amphotericin B and 0.32 μg/mL polymyxin B. The plates placed in an anaerobic jar together with a GENbox microaer paper sachet (Biomerieux, Marcy l'Etoile, France) were incubated to generate a microaerophilic environment containing 5 mL/L oxygen, 10 mL/L CO_2, and 85 mL/L N_2), then the biopsy specimens were incubated at 37℃ for 7 d under a humid condition. *Helicobacter* colonies were identified further by their typical morphology, characteristic appearance on Gram staining, positive urease test. PCR was performed to examine the 16S rRNA, cagA and vacA genes of *Helicobacter* spp. The PCR products were sequenced.

Detection of 16S rRNA, vacA and cagA genes

Approximately 5 mm × 5 mm × 5 mm of tissue was cut and lysed in 0.25% pancreatic RNase, 0.2% collagen enzyme and 0.1 mg/mL proteinase K (Sigma, St Louis). The proteins were extracted with phenol:chloroform, and the genomic DNA was recovered by precipitation with ethanol. Initially, the samples were amplified by *Helicobacter* spp. 16S rRNA primers as previously described[13]: sense primer: 5'-AAC GAT GAA GCT TCT TCT AGC TTG CTA G-3' (28 bp); antisense primer: 5'-GTG CTT ATT CGT TAG ATA CCG TCA T-3' (25 bp) (Shanghai BioAsia Biotechnology Co., Ltd, China). The forward and reverse primer amplified a product of approximately 400 bp. Thirty-five cycles of amplification were performed, each consisting of an initial denaturation at 94℃ for 4 min, followed by denaturation at 94℃ for 1 min, primer annealing at 55℃ for 1.5 min, extension at 72℃ for 2 min, and a final extension step at 72℃ for 10 min. *H pylori* (NCTC11637) was used as the positive control and double-distilled water was used as the negative control. The samples generated a positive result in *Helicobacter* spp. PCR was subsequently performed with another two different sets of primers. A primer pair amplifying a 352-bp product, based on the partial DNA sequence of a species-specific gene encoding *vacA* of *H pylori* was previously described elsewhere[14]. Primers based on *cagA*, amplifying a 297-bp product, were used as previously described [15], including sense primer: 5'-GGA GCC CCA GGA AAC ATT G-3'; antisense primer: 5'-CAT AAC TAG CGC CTT GCA C-3'; sense primer: 5'-ATA ATG CTA AAT TAG ACA ACT TGA GCG A-3'; antisense primer: 5'-TTA GAA TAA TCA ACA AAC ATC ACG CCA T-3'. The *vacA* and *cagA* genes of *H pylori* (NCTC11637) were used as the positive control and double-distilled water was used as the negative control.

Southern hybridization

In order to prove the characteristics of *Helicobacter* spp. 16S rRNA, Southern hybridization was carried out using probes for *Helicobacter* spp., *H pylori*, *H hepaticus*, and *H fennellia* (Beijing AoKe Biotechnology Co. Ltd, China). These probes were designed using Battle software: *Helicobacter* spp. 16S rRNA-cDNA probes: 5'-CGC CGC GTG GAG GAG GAT GAA GGT TTT AGG ATT GTA-3' (36bp): *H pylori* 16S rRNA-cDNA probes: 5'-GAG GGC TTA GTC TCT-3' (15bp); *H hepaticus* 16S rRNA-cDNA probes: 5'-CCT TGC TTG TCA GGG -3' (15bp); and *H fennellia* 16S rRNA-cDNA probets: 5'-CCT TGC TTG ACA GGG-3'(15bp). Hybridization was performed by the above-mentioned probes using the digoxygenin DNA labeling kit (Boehringer Mannheim Company) according to the manufacturer's instructions, then the PCR products were transferred to a nylon membrane (Amersham, Buckinghamshire, United Kingdom) with the capillary blotting technique. The membrane was prehybridized, hybridized, then anti-digoxin (1:5000) and CSPD were added. The bound probes were finally detected by autoradiography after 2 h at room temperature (Kodak Scientific Imaging Film, XK-1 REF 6535009, Rochester, New York).

Sequence analysis of PCR products

The PCR products were purified from agarose gels by the JETsorb DNA extraction kit (Genomed, GmbH, Bad Oeynhausen, Germany). The purified products were sequenced and analysed (Dalian Bao biotechnology Co., Ltd, China). The sequences were compared with the known 16S rRNA of *Helicobacter spp.* and the other bacteria using the GenBank.

Scanning microscopy and in situ hybridization

The appropriate specimens were examined by SEM (JEOL JSM-840). For each hybridization reaction, the biopsy specimens were frozen, cut into 4 μm thick sections using

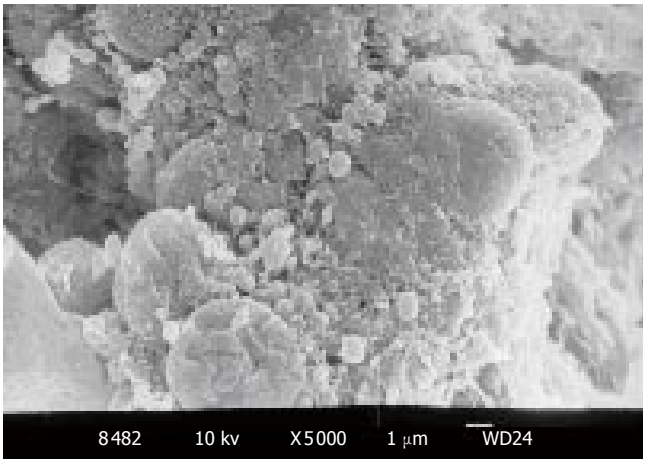

Figure 1 Cocci in adjacent hepatocytes (SEM × 5000).

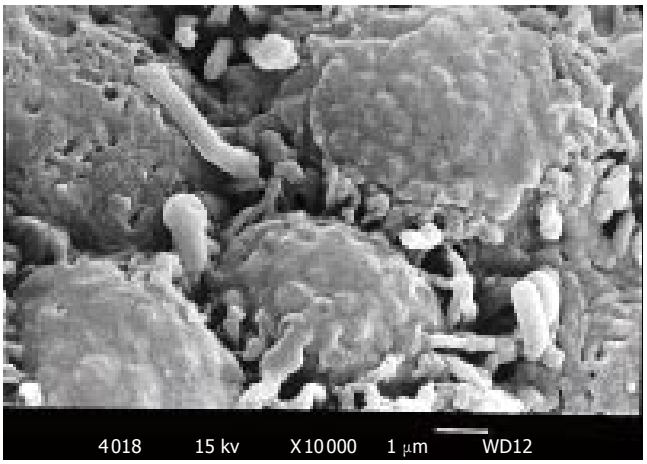

Figure 2 Spiral-shaped bacteria in vicinity of pit cell lineage (SEM × 10 000).

a cryomicrotome (Leica, Wetzlar, Germany) and placed on glass slides. The slides were placed in 4% polyformaldehyde for 20 min, washed and treated with proteinase K (100 μg/mL) at 37℃ for 30 min, washed again and marinated with 0.2% glycin for 10 min, and finally washed with 2 × SSC for 5 min. The sections were covered with 20 μL of 4 kinds of probes mentioned above. The slides were then hybridized overnight at 60 ℃ in a humid chamber and washed with SSC. The sections were incubated in protein blocking buffer. After washed, the slides were incubated with anti-digoxin-alkaline phosphatases complex (1:3000) for 1 h at 37 ℃ and marinated with buffer. After marinated, the slides were counterstained with DAPI overnight, marinated with double-distilled water for 5 min, dehydrated in a graded series of ethanol, hyalined with xylene, covered with balata and observed under microscope.

Statistical analysis

The results of PCR and hybridization were analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of germ culture

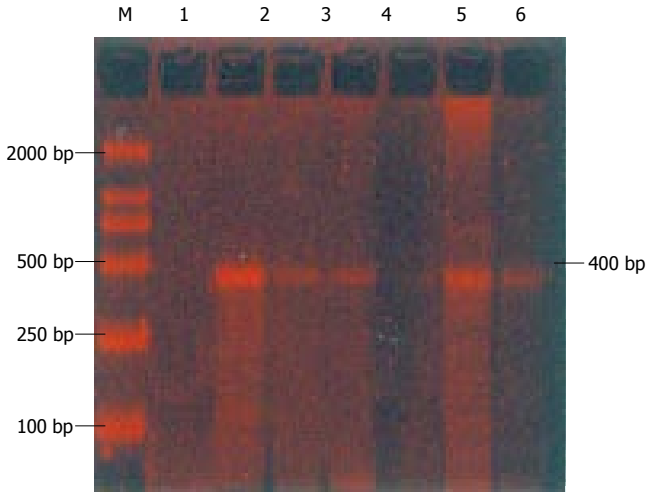

Figure 3 Analysis of *Helicobacter* spp. PCR products from HCC samples. The 400-bp fragments were analyzed by 1.5% agarose gel electrophoresis. Lane M: nucleotide marker; lane 1: negative control (double-distilled water); lane 2: positive control (*H pylori* DNA); Lanes 3, 4, 6: positive samples.

Helicobacter microorganisms were identified by typical appearance on Gram staining, positive urease test and characteristic colony morphology on TEM. After cultured for 48 h, the small and grey colonies were detected in Columbia agar plates, and the mean colony diameter was 1 mm. *Helicobacter* was cultured in 3 HCC and 6 gastric cancer tissue specimens. The positive rate was 10.7% and 24%, respectively. The bacterium was positive in urease test. TEM showed that the bacterium could be divided into two types, one was bacillus with flagellum, the other was coccus with flagellum. All the flagelli were located in one side.

Scanning microscopy

The cocci were observed by SEM in adjacent hepatocytes of 28 HCC samples, and the mean diameter cocci was 0.7 - 0.8 μm (Figure 1). In the 25 gastric cancer samples, 4 were found to have spiral-shaped bacteria (2.0-4.0 μm in length and 0.5-1.0 μm in width). The spiral-shaped bacteria partly adhered to the surface of gastric epithelial cells (Figure 2).

PCR of 16S rRNA,vacA and cagA

The positive rate of HCC and gastric cancer tissue samples was 60.7% and 72%, whereas no sample was positive in the other groups. The size of PCR products corresponded to the expected 400 bp (Figure 3). The positive rate of HCC and gastric cancer tissue samples had no significant difference ($P > 0.05$). Amplified products were identified by Southern hybridization. Besides, the other genes (vacA, cagA) specific for *H pylori* were also detected. The sizes of PCR fragments generated with the vacA (352 bp) and *cagA* primers (297 bp) corresponded to the expected sizes. The cagA gene was detected only in 3 HCC and 2 gastric cancer samples (Figure 4). None of the samples reacted with primers for *vacA* in the two groups.

Sequence analysis and in situ hybridization

The amplified products were sequenced (Figure 5) and compared with *Helicobacter* spp. The *Helicobacter* spp.

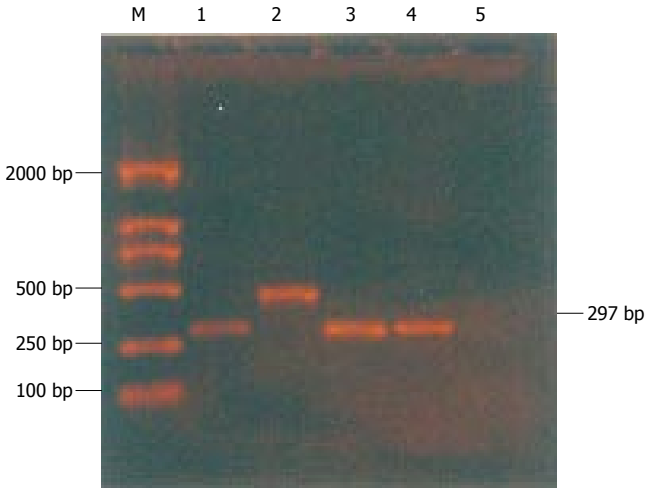

Figure 4 Analysis of *vacA* and *cagA* PCR products from HCC samples. The 352-bp and 297-bp fragments were analyzed by 1.5% agarose gel electrophoresis. Lane M: nucleotide marker; lane 1: positive control (*cagA* DNA); lane 2: positive control (*vacA* DNA); Lanes 3, 4: *cagA* positive samples; lane 5: negative control (double-distilled water).

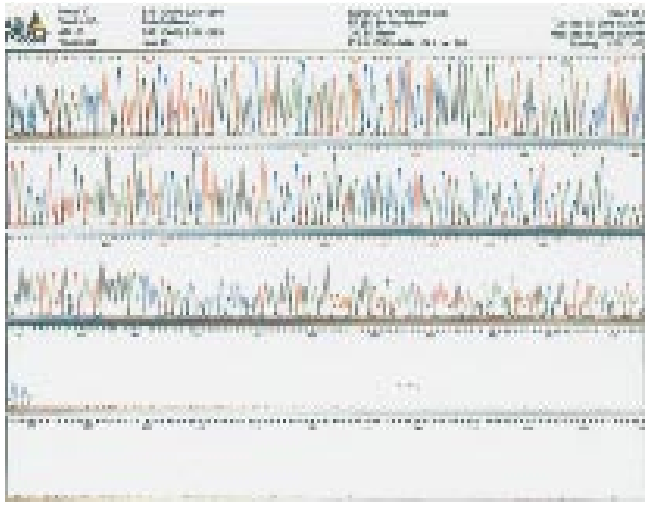

Figure 5 Sequencing results of 16S rRNA in *helicobacter* genus-positive products from HCC.

searched from the GenBank, includeed *H hepaticus, H bilis, H felis, H canis, H muridarum, H mustelae, H nemestrine, H fennelliae, H cinaedi, H acinoyx* (Figure 6). The homology of base pair and amino acid sequence was 97.8% between the amplified products and *H pylori* and 80.9% in *H fennelliae*.

In situ hybridization showed that *Helicobacter* spp. 16S rRNA-mRNA was positive in 17 patients with HCC-with a positive rate of 60.7%. The black-brown granules distributed in cholangioles of hepatocytes were positive hybridization signal. The positive signal was also found in intracellular fluid close to the hepatocyte membrance. All the granules were dispersedly distributed. The number of granules was not different in carcinoma and its adjacent tissues. Seventeen patients with gastric cancer were positive and the positive rate was 68%. The positive granules residing within the epithelium plasma membrane were symmetrically distributed and the number of the granules was not different in carcinoma and its adjacent tissues. *Helicobacter* spp. was negative in patients with colonic cancer (except for one case) and in those with other liver diseases and myoma of uterus.

Table 1 *Helicobacter* detection by different probes

Group	cDNA probes				
	Helicobacter spp.	*H pylori*	*H hepaticus*	*H fennellia*	*P*
HCC	17/28	17/28	0	0	
Gastric cancer	17/25	17/25	0	0	0.40[a]
Other liver diseases	0/22	0	0	0	0
Colonic cancer	1/15	1	0	0	0
Myoma of uterus	0/15	0	0	0	0

[a]$P > 0.05$ *vs* HCC group and gastric cancer group.

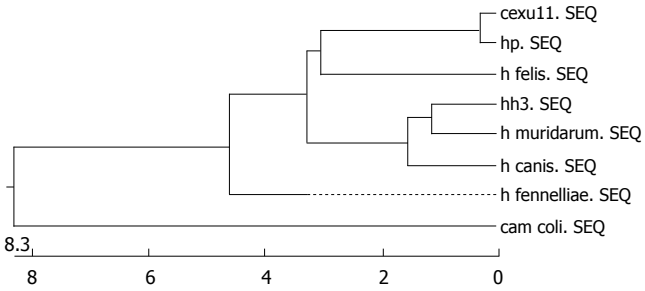

Figure 6 Genic phylogenetic tree of *Helicobacter* spp. and deduced protein.

The positive rate of *Helicobacter* spp. 16S rRNA-mRNA was comparable in HCC and gastric cancer patients with no statistical significance ($P > 0.05$). When HCC and gastric cancer patients were compared with those with other liver diseases, there was a statistical significance($P < 0.01$, Table 1).

In order to confirm the type of *Helicobacter*, the probes for *H pylori*, *H hepaticus* and *H fennellia* were used to detect the positive samples of *Helicobacter* spp. 16S rRNA-mRNA by *in situ* hybridization. The result revealed that the hybridization by *H pylori* probe was consistent with that by *Helicobacter* spp. probe. The positive rate was 60.7% in HCC patients and 68% in gastric cancer patients while being negative in patients with other liver diseases.

DISCUSSION

H pylori is one of the most common bacteria worldwide found in more than 50% of human population[16]. The presence of *H pylori* is the main cause of several gastroduodenal diseases, including peptic ulcer[4,6], gastric cancer[17], and gastric MALT lymphoma[18], and has been designated as class I carcinogen by the World Health Organization[19]. Chronic *H pylori* or *Helicobacter* spp. infection is related with a variety of extragastric diseases, including ischaemic heart disease, liver disease, skin disease, blood disorder and others[8]. The relationship between *Helicobacter* spp. infection and liver disease in humans needs to be further studied. Fan *et al*[20] demonstrated that the seroprevalence of *H pylori* is high in Chinese

patients with HBV-related chronic hepatitis. Experimental infection with *H hepaticus* in mice causes chronic hepatitis and HCC[3]. Thus *H hepaticus* infection of mice provides a uniquely valuable animal model for exploring the mechanisms underlying liver cancer[21]. Avenaud *et al*[11] have demonstrated the presence of genomic sequences of *Helicobacter* spp. in patients with HCC. Ponzetto *et al*[22] reported that the *cagA* gene can be obtained from liver tissue of cirrhotic patients with HCC. By PCR, hybridization and partial DNA sequencing, Nilsson *et al*[10] found that *Helicobacter* genus-specific primers are positive in patients with PBC or PSC. Furthermore, the gene sequence obtained from positive PCR of *Helicobacter* spp. 16S rRNA is usually analogous to *H pylori*. *Helicobacter* spp. has been successfully cultured in liver samples from patients with Wilson's disease by Queiroz and Santos[23]. The isolate is closely related to *H pylori* by biochemical and 16S rRNA analysis. There is evidence that hepatobiliary *Helicobacter* spp. may exist in liver of severe HCV-infected and HCC patients[19]. High rates of human HCC are reported in Southeast Asia, which are caused by hepatobiliary *Helicobacter* spp. and other environmental agents[24]. Pellicano *et al*[25] reported that the presence of genomic sequences of *H pylori* is infrequent in patients who have undergone surgery for metastasis of colon cancer to the liver. In contrast, the presence of genome of *Helicobacter* spp. is higher in patients with HCV-related cirrhosis and HCC. Huang *et al*[26] reported that *Helicobacter* spp. 16S rDNA can be found in patients with primary liver carcinoma. Verhoef *et al*[27] found that gastric colonization with a specific subset of *Helicobacter* strains is associated with the induction of HCC, either directly via colonization of the liver or indirectly via secretion of specific toxins by *Helicobacter* residing in the stomach. Rocha *et al*[28] reported that the presence of *Helicobacter* species DNA in liver is associated with hepatitis C. These observations prompted us to explore a possible association between *Helicobacter* spp. and HCC in Chinese patients.

In this study, the relationship between *Helicobacter* spp. and HCC was investigated by isolation and culture of strains from biopsies, electron microscope, PCR, hybridization techniques and partial DNA sequencing assay. The potential significance of *Helicobacter* spp. in HCC carcinogenesis was investigated. *Helicobacter* spp. was 10.7% (3/28) and 24% (6/25) cultured in HCC and gastric cancer tissue samples, respectively. *Helicobacter* microorganisms were identified by their typical appearance on Gram staining, positive urease test and characteristic colony morphology on transmitting electron microscopy. The bacterium was observed by SEM in the adjacent hepatocytes of HCC samples. The number of cocci was greater than that of bacilli. Our study showed that *Helicobacter* spp. DNA could be found in liver tissue from 60.7% patients with HCC, which might be related to a variety of environmental factors, host characteristic bacteria virulence determinants, and the small size of samples. Eighteen of 25 (72%) liver samples from patients with gastric cancer were positive by PCR analysis using *Helicobacter* spp. primers. Patients with HCC and gastric cancer had a higher positivity with no statistical significance ($P > 0.05$), suggesting that *Helicobacter* spp. infection is closely related with diseases of the digestive system. Amplified products were identified by Southern hybridization, suggesting that *Helicobacter* spp. infection might occur in HCC patients. Sequencing showed that PCR-amplified products and *H pylori* had a 97.8% homology. To make sure that the bacteria was *H pylori*, the vacuolating cytotoxin gene A (*vacA*) and cytotoxin-associated gene A (*cagA*) specific for *H pylori* were also detected by PCR. *H pylori* can be divided into $cagA^+$- *H pylori* and $cagA^-$-*H pylori* in clinical medicine, leading to a different result in gastrointestinal disease. $CagA^-$-*H pylori* usually colonizes the mucous gel or the apical epithelial surface, whereas $cagA^+$- *H pylori* colonizes the immediate vicinity of epithelial cells or the intercellular spaces[29]. Strains possessing the *cag* pathogenicity island are more likely to cause disease rather than those lacking this locus[30]. The *vacA* and *cagA* help gastric epithelial cells to form vacuolation, injury, necrosis, ulcer, etc. *H pylori* strains possessing *cagA* are associated with the development of peptic ulcer, gastric cancer and extragastric diseases. Most of the strains could be divided into two major types. Type Ⅰ bacteria have the gene coding for cagA and express CagA and VacA. Type Ⅱ bacteia do not express either cagA or vacA[11]. Therefore, the difference between type Ⅰ and type Ⅱ bacteria is due to toxicity. In our study, the cagA gene was detected in only 3 HCC and 2 gastric cancer samples (Figure 4). None of the samples reacted with primers for vacA in the two groups. As for the genotype of *H pylori*, type Ⅱ bacteria have preference over type Ⅰ, which is consistent with the report of Avenaud *et al*[11].

To confirm the *Helicobacter* spp. distribution in HCC patients, *Helicobacter* spp. 16S rRNA-mRNA was detected by *in situ* hybridization with a positive rate of 60.7%, which was coincident with the result by PCR. Besides, the positive rate in gastric cancer patients was 68%. The positive granules residing within the epithelium plasma membrane were distributed symmetrically. *Helicobacter* spp. was negative in patients with colonic cancer except for one case and those with other liver diseases and myoma of uterus. The positive rate in HCC and gastric cancer patients had no statistical significance ($P > 0.05$). When HCC patients were compared with those with other liver diseases, there was no statistical significance ($P < 0.01$).

In conclusion, *Helicobacter* spp. infection is related with diseases of the digestive system, especially HCC and gastric cancer. Further studies are needed to establish the role of *H pylori* in HCC.

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Recombinant hybrid protein, Shiga toxin and granulocyte macrophage colony stimulating factor effectively induce apoptosis of colon cancer cells

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Abstract

AIM: To investigate the selective cytotoxic effect of constructed hybrid protein on cells expressing granulocyte macrophage colony stimulating factor (GM-CSF) receptor.

METHODS: HepG2 (human hepatoma) and LS174T (colon carcinoma) were used in this study. The fused gene was induced with 0.02% of arabinose for 4 h and the expressed protein was detected by Western blotting. The chimeric protein expressed in *E.coli* was checked for its cytotoxic activity on these cells and apoptosis was measured by comet assay and nuclear staining.

RESULTS: The chimeric protein was found to be cytotoxic to the colon cancer cell line expressing GM-CSFRs, but not to HepG2 lacking these receptors. Maximum activity was observed at the concentration of 40 ng/mL after 24 h incubation. The IC_{50} was 20 ± 3.5 ng/mL.

CONCLUSION: Selective cytotoxic effect of the hybrid protein on the colon cancer cell line expressing GM-CSF receptors (GM-CSFRs) receptor and apoptosis can be observed in this cell line. The hybrid protein can be considered as a therapeutic agent.

Key words: Shiga toxin; hGM-CSF; Apoptosis; Colon cancer; Cell lines

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INTRODUCTION

Although systemic treatment of colon cancer including combination of surgery, hormone therapy, chemotherapy is commonly believed to be effective in prolonging patient survival, colon cancer remains one of the principal cancer-related deaths[1]. Human cancer is becoming more definable by cell surface proteins. One of these molecules is GM-CSF receptor that expresses in several tumors including colon cancer[2].

GM-CSF is used to attenuate the myelosupressive effects of chemotherapy in the treatment of hematologic malignancies and solid tumors, but GM-CSF usually does not stimulate growth of solid tumors[2]. It was reported that GM-CSFR is absent on most immature hematopoietic progenitors but increases expression during maturation[3]. This finding strongly suggests that GM-CSFR may be a useful target for recombinant toxins or immunotoxins.

Immunotoxin comprises cells targeting and killing moieties. Therefore, bacterial or plant toxins are used as the killing moieties. Shiga and Shiga-like toxins (STX, SLTs) are ribosome-inactivating proteins (RIPs) produced by *Shigella* and *E.coli*, composed of an enzymatic A subunit non-covalently associated with a pentamer receptor-binding subunit. SLTs inhibit protein synthesis in eukaryotic cells by releasing adenine residue from the highly conserved aminoacyl-tRNA-binding site which exists on large subunit of ribosome [4]. In this study, the catalytic domain of Shiga toxin (StxA1) and hGM-CSF were genetically fused and expressed in *E.coli*. Then, the function of recombinant protein was assessed on colon cancer cell line.

MATERIALS AND METHODS

Preparation of purified recombinant chimeric protein (StxA1-GM-CSF)

The catalytic domain of Shiga toxin, StxA1, was fused

teria with 0.02% arabinose for 4 h incubation. The expressed protein was purified following the manufacturer's instructions (Invitrogen, USA). The amount of protein was estimated by Bio-Rad protein assay kit (Bio-Rad, USA).

Cell culture

HepG2 cell line derived from human hepatoma and LS174T cell line derived from colon carcinoma were provided by Cell Bank of Institute of Development, Aging and Cancer, Tohoku University, Japan. These cell lines were kept in RPMI-1640 medium (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (Gibco-BRL , Germany) at 37 ℃ in 50 mL /L CO_2 atmosphere.

Cytotoxicity assay

The cytotoxic effect of SltxA1-GM-CSF was determined by trypan blue exclusion assay and MTT[5] assay. For trypan blue dye exclusion assay, 2.5×10^4 cells were seeded in one well of 96-well plates and different concentration (10-160 ng) of SltxA1-GM-CSF was added to each well. After incubation for 24, 48, and 72 h, the number of viable cells was determined.

For MTT assay, HepG2 and LS174T cell lines were cultured and StlxA1-GM-CSF was added as described above. After 24, 48 or 72 h incubation, 10 μL of 0.5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Japan) was added to each well and further incubated at 37 ℃ in 50 mL/L CO_2 atmosphere for 4 h to allow MTT to be converted to formazon crystals by reacting with metabolically active cells. Reactions were stopped by addition of 10% SDS in 0.01 mol/L HCl and absorbance at 570 nm was determined.

Neutral comet assay

Neutral comet assay was carried out as previously described[6] with some modifications. Briefly, freshly prepared cell suspension (2×10^3 / 10 μL) was mixed with 150 μL of 1% low-melting agars. The mixture was layered on top of the microscopic slide coated by 1% agarose. After low melting agarose was solidified in a refrigerator for 10 min, the slide was gently immersed in a freshly prepared lysing solution (2% SDS, 0.03 mol/L EDTA) for 30 min protected from light. After the slides were washed with TBE buffer, electrophoresis was carried out at 25 V for 25 min. Then, comets were visualized with 1 mmol/L propodium iodide. One hundred and fifty cells per slide were analysed under fluorescent microscope.

Hoechst staining

After administration of SltxA1-GM-CSF, cells were fixed with 1% paraformaldehyde for 30 min, washed with PBS and stained with 1mmol/L Hoechst 33342 (Sigma, Japan) for 10 min. Nuclear morphology of at least 400 cells was randomly observed under fluorescent microscope.

Statistical analysis

The results were expressed as mean ± SD and each value represented the mean of three experiments. Differences between groups were compared using the Student's *t* test. $P < 0.05$ was taken as significant.

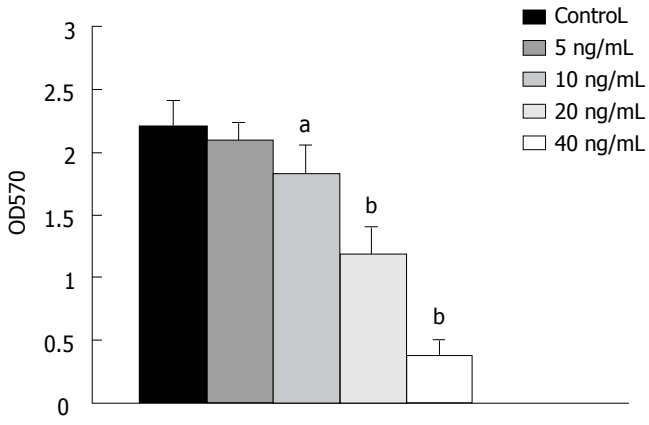

Figure 1 Effect of different concentrations of StxA1-GM-CSF on LS174T cell line by MTT assay. ($^aP < 0.01$, $^bP < 0.001$ *vs* trypan blue exclusion assay).

RESULTS

Expression and characterization of StxA1-GM-CSF

The fusion protein consisting of 1-255 a.a. of A1, the catalytic domain of Shiga-toxin and 1-127 a.a. of hGM-CSF was observed. The recombinant was analysed by SDS-PAGE and Western blot as previously described[7].

Cytotoxicity

Cytotoxicity of hGM-CSF to cells was analysed by both trypan blue exclusion assay and MTT assay. LS174T cells showed the extreme susceptibility to StxA1-GM-CSF (Figure 1), while HepG2 cells lacking GM-CSFR showed very poor susceptibility to StxA1-GM-CSF. Maximum activity was observed at the concentration of 40 ng/mL ($P < 0.001$) after 24 h incubation. Higher concentration and longer treatment time did not show any profound effect. The IC_{50} was 20 ± 3.5 ng/mL.

Apoptosis

We next examined death of LS174T cells induced by the StxA1-GM-CSF by comet assay. In comet assay apoptotic cells after treatment showed puffy tails and pin heads while normal cells had spherical heads but no tails (Figure 2). To verify the apoptotic results obtained by comet assay, cells were analyzed by Hoechst 33342 staining. Apoptotic cells with nuclear condensation and fragmentation were analyzed (Figure 3). The results indicated that the apoptotic cells were effectively induced by the toxin (Figure 4).

DISCUSSION

In this study we constructed the recombinant protein of StxA1-GM-CSF by overlap extension PCR, which was expressed in pBAD/gIIIA system[7]. Al-Jaufy *et al*[8] have also made the recombinant protein genetically. They fused StxA with HIV gp120-binding domain of CD4 by restriction enzyme sites. However, some limiting factors like incomplete digestion, absence of suitable restriction sites for genes of interest and appropriate vector have limited the

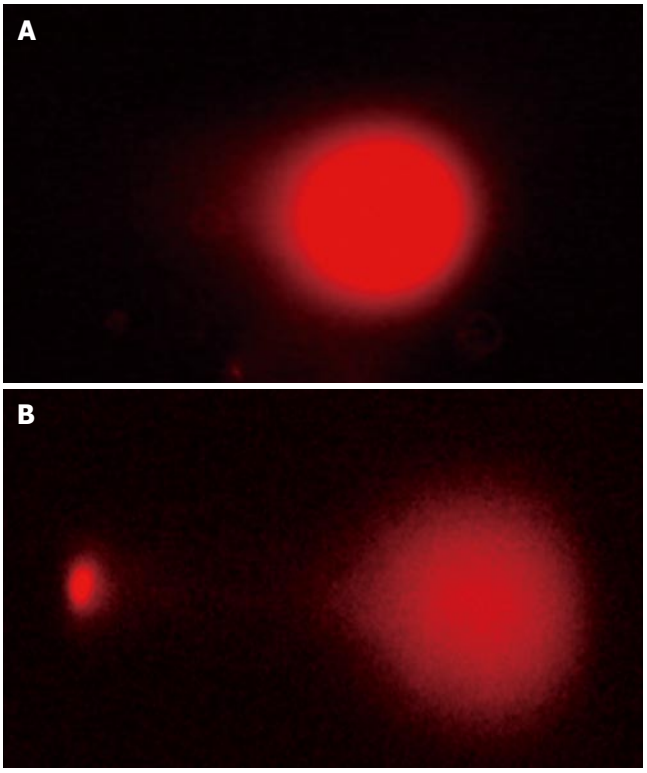

Figure 2 Morphology of normal cells (**A**) and apoptotic cells (**B**) after treatment with hybrid protein.

application of this method. Accuracy and ease of manipulation, are likely the advantages of PCR technique.

StxA is an established inhibitor of eukaryotic translation and more potent than ricin[9]. No reduction in its enzymatic inhibitory activity has been reported when it is genetically fused with HIV gp120-binding domain of CD4[8]. Similarly in our study, fusion of StlxA1 with hGM-CSF caused no reduction in its inhibitory effect. In addition, the enzymatic domain of diphtheria toxin has been fused to hGM-CSF using recombinant DNA techniques and is under clinical trial[10]. Our results suggest that StxA1-GM-CSF is more toxic than DT-GM-CSF, since IC_{50} is 20 ± 3.5 ng/mL for StxA1-GMCSF and 70 ± 18 ng/mL for DT-GM-CSF[8]. In this study, LS174T showed very obvious cytotoxic effect and changes characteristic of apoptosis were observed. A close correlation between protein synthesis inhibition and apoptosis induction has also been reported for DT-GM-CSF in which LS174T is not sensitive to apoptosis while other cell lines are more sensitive to cytotoxicity and apoptosis[11].

Agarose gel electrophoresis is one of the commonly used techniques for the detection of apoptotic cells. This technique usually involves a DNA isolation procedure from millions of cells and obtained results can not be quantified[12]. TUNEL assay is an established method for the detection of apoptotic cells, but this method is associated with a number of artefacts[13]. Annexin V labelled with fluorescent protein like FITC is used for rapid cytofluorometric analysis of apoptosis, but this method can produce false positive results when membrane is damaged[14]. Comet assay is sensitive, simple and fast. Usually this method is used to detect DNA damage like single or double strand breaks. On the other hand, this method also can detect single apoptotic cells in a large number of cells[15]. A few studies have tried to delineate the mechanism of apoptosis induction by Stx[16-18]. More knowledge about its mechanism can help us prevent shigellosis and enable us to tackle the problem of resistance to cancer therapy. Induction of apoptosis by hybrid protein indicates that catalytic domain of Shiga toxin in hybrid protein can not activate the cellular apoptosis machinery directly since the toxin inactivates ribosomes that inhibit protein synthesis.

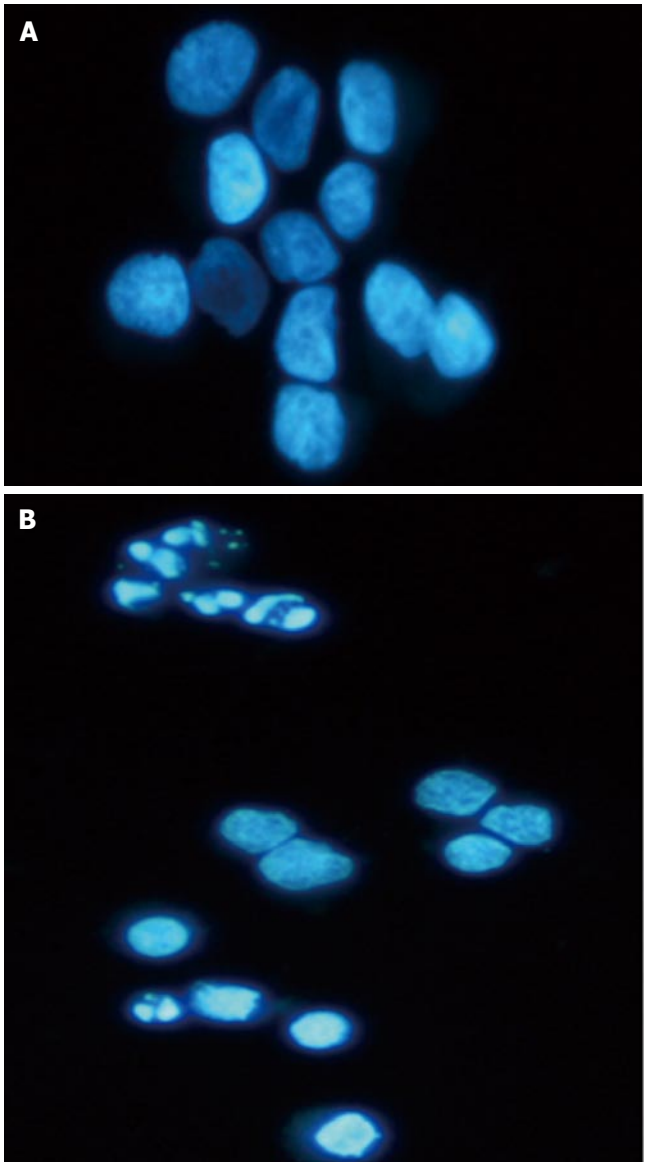

Figure 3 Hoechst 33342-staining of LS174T cells treated with StxA1-GM-CSF. **A**: control; **B**: cells after treatment.

In conclusion, more investigations are required to clarify how StxA1-GMCSF induces apoptosis. Our results reveal that the hybrid protein is toxic to the colon cancer cell line and can be considered as a therapeutic agent.

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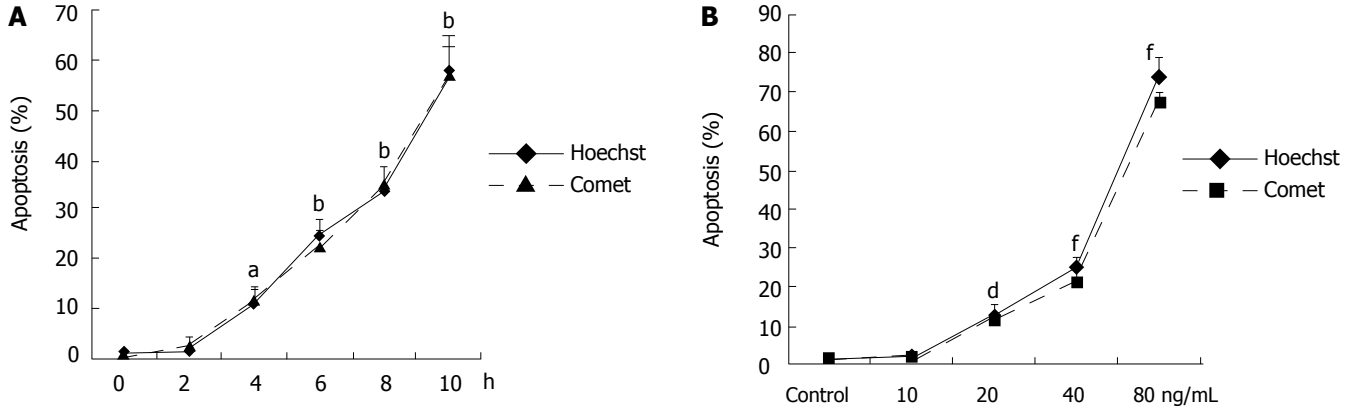

Figure 4 Apoptosis percentage in the presence of 40 ng/mL StxA1-GM-CSF at different time intervals (**A**), $^{a}P<0.05$, $^{b}P<0.001$. Apoptosis percentage in the presence of different concentration of StxA1-GMCSF for 6 h (B). $^{d}P < 0.01$, $^{f}P < 0.001$.

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BASIC RESEARCH

Three-dimensional MR and axial CT colonography *versus* conventional colonoscopy for detection of colon pathologies

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Abstract

AIM: To evaluate the sensitivity and specificity of MR colonography (MRC) and CT performance in detecting colon lesions, and to compare their sensitivity and specificity with that of conventional colonoscopy.

METHODS: Forty-two patients suspected of having colonic lesions, because of rectal bleeding, positive fecal occult blood test results or altered bowel habits, underwent the examinations. After insertion of a rectal tube, the colon was filled with 1000-1500 mL of a mixture of 9 g/L NaCl solution, 15-20 mL of 0.5 mmol/L gadopentetate dimeglumine and 100 mL of iodinized contrast material. Once colonic distension was achieved, three-dimensional gradient-echo (3D-GRE) sequences for MR colonography and complementary MR images were taken in all cases. Immediately after MR colonography, abdominal CT images were taken by spiral CT in the axial and supine position. Then all patients were examined by conventional colonoscopy (CC).

RESULTS: The sensitivity and specificity of MRC for colon pathologies were 96.4% and 100%, respectively. The percentage of correct diagnosis by MRC was 97.6%. The sensitivity and specificity of CT for colon pathologies were 92.8%, 100%, respectively. The percentage of correct diagnosis by CT was 95.2%.

CONCLUSION: In detecting colon lesions, MRC achieved a diagnostic accuracy similar to CC. However, MRC is minimally invasive, with no need for sedation or analgesics during investigation. There is a lower percentage of perforation risk, and all colon segments can be evaluated due to multi-sectional imaging availability; intramural, extra-intestinal components of colonic lesions, metastasis and any additional lesions can be evaluated easily. MRC and CT colonography are new radiological techniques that promise to be highly sensitive in the detection of colorectal mass and inflammatory bowel lesions.

Key words: MR colonography; CT colonography; Colorectal mass; Inflammatory bowel disease; Conventional colonoscopy

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INTRODUCTION

The colon is the end part of the gastrointestinal tract from the ileocecal valve to the anal region. For many years, barium examination and endoscopy were the only proven diagnostic methods for evaluating diseases of the colon. In the last 15-20 years, however, spiral computed tomography (CT) has also been shown to be an essential tool in radiological evaluation of the gastrointestinal tract. CT and MRI can be used to evaluate diseases of the colon. They can show the colon wall, colon lumen and the adjacent tissues and organs. CT and MRI are particularly useful in the initial staging of colon neoplasms, assessing the response of colon tumors to therapy and postoperative recurrence of gastrointestinal tumors, evaluating possible causes for gastrointestinal organ displacement and extrinsic impressions detected by barium studies or endoscopy. Moreover, CT and MRI are useful for the detection of inflammatory bowel disease[1-5]. Selective three-dimensional (3D) imaging of the colon was first described in 1994 by Vinning *et al*[6] as a method using spiral CT to provide a computer-simulated endoluminal perspective of the air distended colon. In 1997, Luboldt *et al*[8] first described 3D imaging of the colon filled with paramagnetic contrast as MR colonography[6-8].

Colorectal cancer is the third most common cancer and the second leading cause of cancer-related death in western countries. Most colorectal cancer evolves from pre-existing adenomatous polyps. The incidence of

colorectal cancer could be considerably reduced if polyps and small tumors were detected and eliminated prior to their malignant degeneration[9-11]. There is ongoing research for a colorectal cancer screening test that is cost-effective, safe, and acceptable to patients. Current screening methods for colorectal polyps and colonic cancer include fecal occult blood testing, sigmoidoscopy, colonoscopy and double contrast barium enema examination. The effectiveness of each modality as a screening tool remains controversial, and each method has inherent limitations. MR colonography based on MR imaging is a relatively new diagnostic modality for diagnosing colon pathology.

The purpose of this prospective study was to evaluate the sensitivity and specificity of MR colonography and CT in 42 patients who were suspected of having colonic lesions. Standard colonoscopy and histopathologic examination were accepted as the reference standard.

MATERIALS AND METHODS

Patients

A total of 42 patients (25 men, 17 women; mean age 59.3, range 2-85 years), who were suspected of having colonic lesions because of rectal bleeding, positive fecal occult blood test results or altered bowel habits, underwent MR colonography and CT examination, followed by a conventional colonoscopy (CC). All patients underwent standard bowel preparation 24 h before examination. All patients gave written informed consent, and the procedures were approved by the Local Ethics Committee.

Methods

MR colonography was performed on a 1.5T MR system (Edge, Picker, USA). No sedative or analgesic agents were used. Patients were placed in a supine position on the MR table. After insertion of a rectal tube, the colon was filled with 1000-1500 mL of a mixture of 0.9 g/L NaCl solution (1000 mL) and 0.5 mmol/L gadopentetate dimeglumine (15-20 mL) and 300 g/mL iodinized contrast material (100 mL). When the contrast material reached the cecum, the 3D colon imaging data were acquired using a T1W 3D Gradient-Echo Sequence (GRE) (TE: 2.49 ms, TR: 6 ms, flip angle: 10, thickness: 2.5 mm, FOV: 40 cm-43 cm, matrix: 128 × 192). Further sequences were performed on all patients of axial spin-echo (SE) T1W (TE: 10 ms, TR: 130 ms, flip angle: 90, thickness: 8 mm, FOV: 40 cm-43 cm matrix: 192 × 256), and fatsat SE T1W (TE: 20 ms, TR: 749 ms, flip angle: 90, thickness: 7 mm, FOV: 40 cm-43 cm, matrix: 192 × 256).

Immediately after the MR colonography, abdominal CT images were taken on the spiral CT (PQS, Picker, USA) in the axial planes (kV: 130, mA: 175, thickness: 5 mm, matrix: 512 × 512) and supine position. The three-dimensional MR data sets were analyzed in the multiplanar reformation and evaluated completely and separately and independently by two experienced radiologists. The CT axial plane images were evaluated by both the experienced radiologists. Each radiologist recorded the location and the size of colorectal masses or defined the colon lumen, wall and adjacent tissues to lesions of the large bowel, respectively. If their interpretation of the MR colonography and CT images differed, consensus was reached by review and discussion of the controversial images.

All patients were examined with the same endoscopist video (Pentax EC 38 40 TL, Tokyo, Japan) colonoscope. The location and size of any endoluminal lesions were identified and the colon wall pathologic appearance was recorded. All lesions found by CC were biopsied or removed by polypectomy. All specimens were examined histologically for differential diagnosis of inflammatory disease, hyperplastic polyps, adenomatous polyps, and cancers.

Standard colonoscopy and histopathologic examination were accepted as the references, so the MR colonography and CT were being evaluated for sensitivity, specificity and correct diagnosis ratio in the detection of colonic lesions. Each MRC examination lasted about 20-30 min, CT examination about 10-15 min and CC examination about 20-30 min. MR colonography and CT were well tolerated by all patients with no post-procedural complications after MR colonography, CT or CC.

RESULTS

A total of 42 patients suspected of having colonic lesions underwent MR colonography and abdominal spiral CT. Colonic lesions were identified by MR colonography in 26 patients. MR colonography was normal in 16 patients. On the basis of MR colonography and CT, 17 colon carcinoma (65.3%), 2 invasion to rectum (7.7%), 1 recurrent colon tumor (3.8%), 4 inflammatory bowel disease (15.3%), 1 hirschsprung disease (3.8%), and 1 diverticulosis (3.8%) were determined and these lesions were confirmed by conventional colonoscopy and histopathologic examination.

MR colonography and CT identified colorectal cancer in 17 patients. Malignant tumors of the colon were located in the rectum (6), rectosigmoid region (3), caecum (2), ascending colon (1) (Figure 1), descending colon (1) and sigmoid colon (4) (Figure 2). Malignant tumors of the colon appeared on MR colonography and CT as a tumor mass projecting into the lumen of the colon or as an asymmetrical or circumferential thickening of the bowel wall with deformation and narrowing of the lumen. Fourteen adenocarcinoma, two mucinous adenocarcinoma and one tubulovillous adenoma (carcinoma *in situ*) were confirmed by histopathologic examination. Seventeen patients with colon tumors underwent CC. A complete CC was achieved in 14 patients. In three patients, CC could not be evaluated completely due to occlusive carcinoma. However, in these patients, all of the colon segments were examined by MR colonography. The results of the MR colonography and CT were compared with the colonoscopy, histopathologic examination and surgery results. All patients with colon tumors had been correctly identified on MR colonography and CT.

Four patients with inflammatory bowel disease were correctly identified on MR colonography and CT. These were identified by histopathologic examination as ulcerative

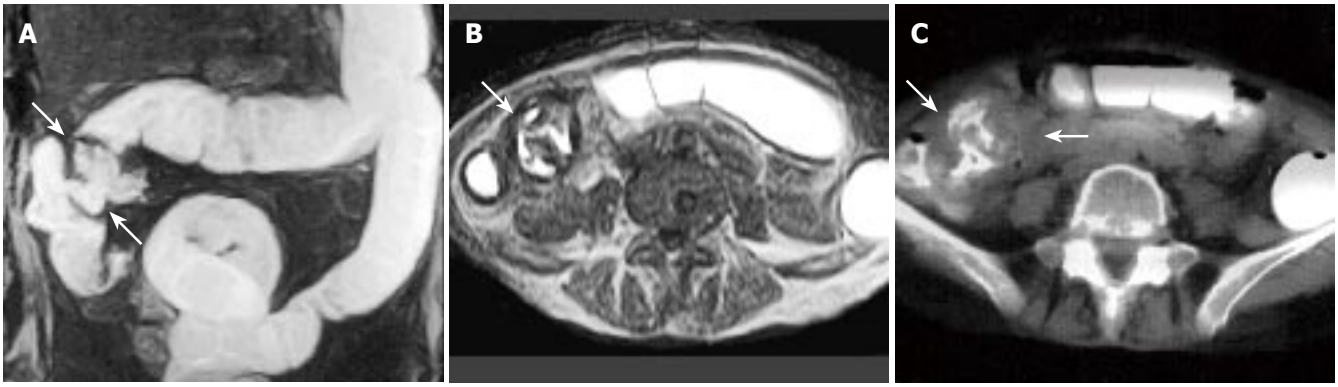

Figure 1 A 59-year-old woman with cecum and ascending colon carcinoma. **A**: MR colonography MIP (maximum intensity projection) image showing asymmetrical, irregular wall thickening at the cecum and ascending colon segments (arrows); **B**: SE T1W axial image; **C**: axial CT image showing asymmetrical, irregular wall thickening of the ascending colon (arrows).

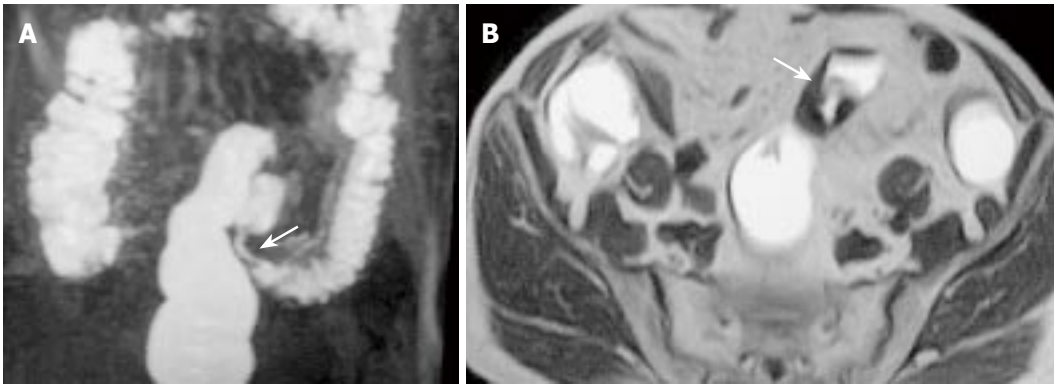

Figure 2 A 77-year-old man with sigmoid carcinoma. **A**: MR colonography MIP (maximum intensity projection) image showing asymmetrical, annular wall thickening at the sigmoid colon segment (arrow); **B**: SE T1W axial image showing asymmetrical, irregular wall thickening of the sigmoid colon (arrow).

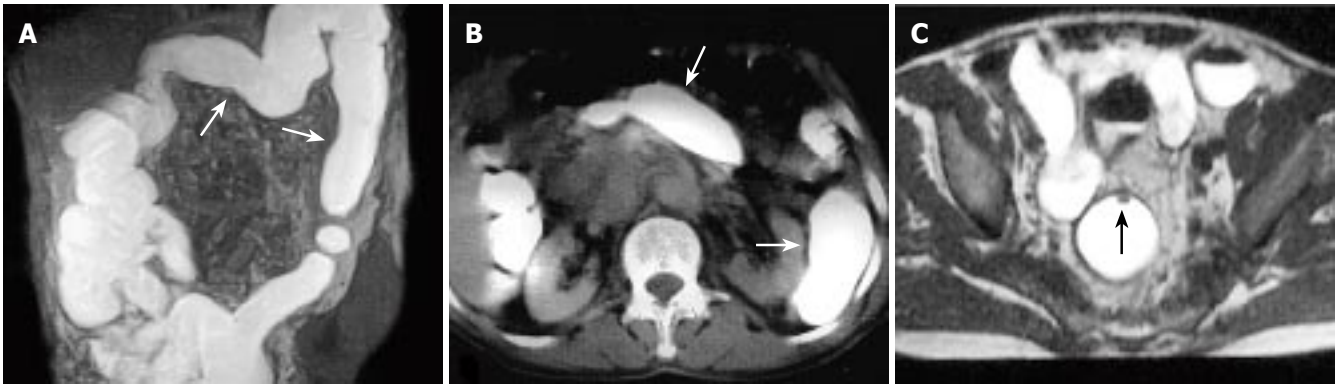

Figure 3 A 63-year-old man with ulcerative colitis. **A**: MR colonography MIP (maximum intensity projection) image; **B**: axial CT image showing haustral flattening at transverse and descending colon segments (arrows); **C**: SE T1W axial image showing a 0.5-cm polyp at the rectosigmoid junction (arrow).

colitis (Figure 3), non-specific colitis and ileocaecal region inflammatory disease (Figure 4). In one patient non-specific colitis was evaluated by histopathologic examination and colonoscopy, but it was not identified by MR colonography and CT. Thus MRC and CT failed to identify 20% (1/5) patients with inflammatory colon disease. In 2 patients with colon carcinoma, the colonoscopy detected polyps (size: 3 mm in one patient and 7 mm in the other) which had not been diagnosed with MR colonography. In one of them, the MR examination had been insufficient because of technical reasons. In the other patient, the small polyp was not identified from the adjacent mass. In the 2 patients with colon carcinoma and one patient with ulcerative colitis, colonoscopy detected polyps (size: 3 mm in 1 patient, 5 mm in 1 patient and 7 mm in the other) which had not been diagnosed with CT. In all three cases, the CT examination had been insufficient because of technical reasons and the small polyps had not been identified from the adjacent mass.

MR colonography and CT identified invasion to the rectum in two patients. In one case where there was bladder cancer invasion to the rectum, both the MR colonography and CT examinations identified invasion to the rectum and further MR colonography showed the

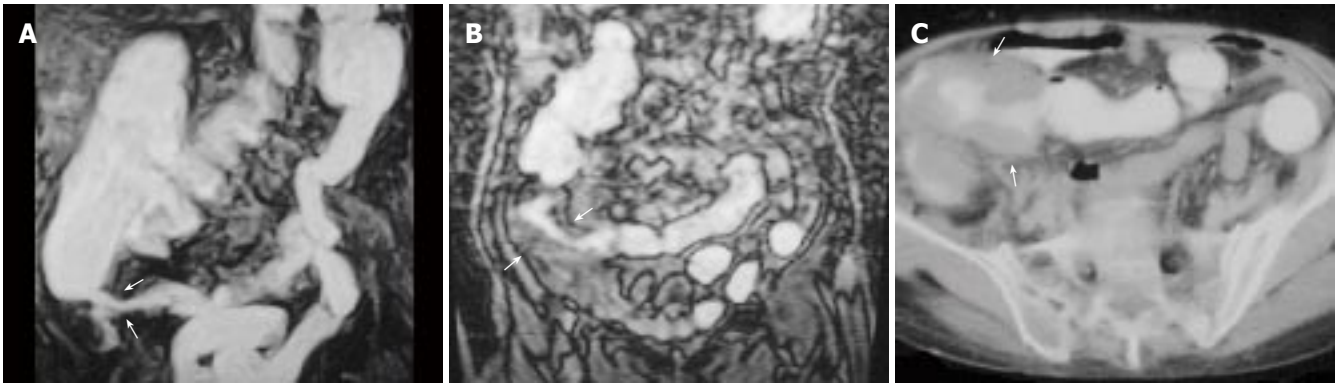

Figure 4 A 70-year-old man with inflammatory disease in the ileocaecal region. **A**: MR colonography MIP (maximum intensity projection) image; **B**: coronal plane MR colonography raw data image; **C**: axial CT image showing asymmetrical, irregular wall thickening of the ileocaecal region (arrows) and serpiginous strands extending into soft tissue due to inflammatory disease.

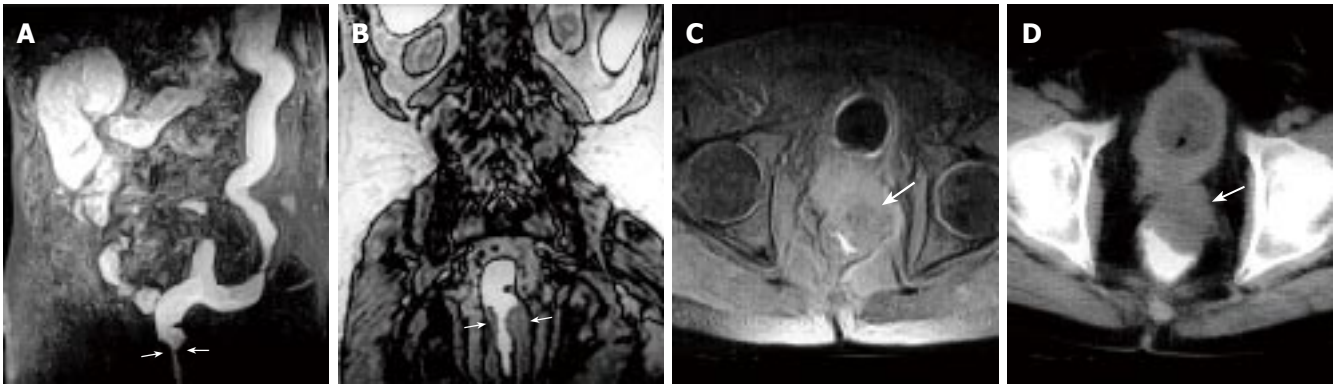

Figure 5 A 63-year-old man with rectum carcinoma invasion to prostate. **A**: MR colonography MIP (maximum intensity projection) image showing luminal narrowing at rectum (arrows); **B**: MR colonography raw data coronal image showing luminal narrowing due to annular thickening of the rectum wall (arrows); **C**: SE axial T1W image; **D**: axial CT image showing asymmetric wall thickening at rectum (arrows), and invasion to the fatty tissue arround the mass.

presence of rectovesical fistula due to bladder cancer. In the other patient, an invasion to the rectum due to prostate cancer was detected (Figure 5). The sensitivity and specificity of MR colonography for colon pathologies were 96.4% and 100%, respectively. The percentage of correct diagnosis of MR colonography was 97.6%. The sensitivity and specificity of CT for colon pathologies were 92.8% and 100%, respectively. The rate of correct diagnosis of CT was 95.2%. The MR colonography was well tolerated without sedation or analgesia and no complications were observed. MR colonography and CT identified 19 extracolonic lesions in 12 of 42 patients. These lesions were liver metastases, hydatic cyst of the liver, simple cyst of the liver, mesenteric cyst, gallbladder carcinoma, duodenum carcinoma, renal cyst, gastric tumor, multiple lymphadenopathy, intraperitoneal lipoma, hiatal hernia, subcapsular hematoma of the spleen and pleural effusion.

DISCUSSION

Colorectal cancer is the second leading cause of cancer-related death. Most colorectal cancer evolves from adenomatous polyps and screening for colorectal polyps with subsequent polypectomy has been shown to constitute an effective approach to decreasing its incidence[9,10]. However, as evidenced by disappointing participation in colorectal screening and the continuing high incidence of colorectal cancer, new screening strategies may prove beneficial. To prove effective in reducing mortality from colorectal cancer, new screening methods must demonstrate a high diagnostic accuracy at a low cost, and be proven safe and highly acceptable to patients[12].

Fecal occult blood testing has a sensitivity of less than 10% for adenomatous polyps, and a sensitivity of less than 15% for the detection of polyps under 2 cm in size[13]. In contrast, the promise of MR colonography is to detect malignant and premalignant polyps with a sensitivity rivaling colonoscopy.

Flexible sigmoidoscopy allows for the examination of only the distal 60 cm of the colon which limits evolution to the descending colon, sigmoid, and rectum; inevitably, lesions are missed in more than half the subjects who have advanced colonic adenoma located proximal to the splenic flexure but who do not have a distal index polyp[14,15]. Colonography, in contrast, images the entire colorectum and may be able to decrease the mortality by detecting more right-sided lesions. In retrospective evaluations of double-contrast barium enema examination, investigators have found sensitivities of 71%-95% for the detection of colorectal cancer[16]. However, in prospective studies of double-contrast barium enema examination, data have

shown the sensitivities as low as 50%-75% for colorectal cancer detection in asymptomatic patients with positive fecal occult blood test[17]. A recent study in which double-contrast barium enema examination was compared with colonoscopy for colonic surveillance after polypectomy found a poor detection rate of 48% for polyps ⩾10 mm in size, as well as a poor overall detection rate of only 39% for adenomas[18]. Unlike barium enema, MR colonography does not suffer from superimposition and can explain the attenuation characteristics of suspicious lesions, as well as allowing for the evaluation of pericolonic tissues.

In most centers, colonoscopy has emerged as the principal means of examining the colon. Although standard colonoscopy is a total colonic examination that allows lesion biopsy and resection, it fails to demonstrate the entire colon in up to 5% of cases examined by an experienced gastroenterologist[19], and up to 20% of all adenomas are missed[20].

Furthermore, there is a risk of complications associated with diagnostic and therapeutic colonoscopy, including perforation (1 in 1000), major haemorrhage (3 in 1000), and death (1 in 30 000)[12,20]. In addition, colonoscopy is limited by poor patient acceptance, which is a most important variable for a screening test[21,22]. Rex *et al*[23] have shown that even when it is offered free of charge, most patients refused to undergo colonoscopy for primary colorectal cancer screening.

In colorectal cancer screening, MR colonography can play an important role for patients who have undergone incomplete endoscopic colonoscopy. Common reasons for incomplete colonoscopy are redundant bowel loops and occlusive carcinoma. MR colonography can achieve a complete examination of the colon in these patients. In patients with occlusive carcinoma, the evaluation of the proximal colon is necessary to exclude a secondary neoplasia, which occurs in 5% of these cases[24]. MR colonography is available for detecting colorectal masses. Luboldt *et al*[25] performed MR colonography in 132 patients referred for CC, showing a sensitivity of 93% and specificity of 99% of MR colonography. A similar study by Pappalardo *et al*[26] compared MR colonography with conventional colonoscopy in 70 patients. All patients who underwent MR colonography had satisfactory studies and MR colonography achieved a diagnostic accuracy similar to that of conventional colonoscopy (sensitivity of 96%, specificity of 93%).

MR colonography techniques have recently been introduced as potential methods for colorectal screening. MR colonography may have a role in accurately staging colorectal cancers, in particular if combined with state-of-the-art MR imaging of the liver. In the same manner as for staging, MR colonography can also be used for post-operative surveillance[27]. Besides the detection and assessment of neoplastic disease, MR colonography can also be employed for the evaluation of inflammatory bowel disease. Over the past decade, several authors have found that MR imaging is a useful, non-invasive tool in patients with Crohn's disease, regardless of whether it is manifested in the small bowel, large bowel, or in the perianal region[28-30]. MR imaging can be used to assess disease activity and may help distinguish reversible inflammatory changes from irreversible fibrostenosis[31-33]. In contrast to double-contrast barium enema or conventional colonoscopy, MR colonography might be used to distinguish the Crohn's strictures that require surgery from those that might benefit from anti-inflammatory therapy, in addition to visualizing the colon proximal to a narrowing, and assessing extracolonic complications of the disease, including fistula and abscesses[34].

An enema with dilute iodinated contrast material can be administered well by the rectal tube in advance of the CT study, just prior to imaging. CT can also be used to evaluate the colon wall. In its characterization of colon neoplasms, CT is useful to categorize the extent of tumor as follows: intraluminal mass without wall thickening; wall thickening focal or diffuse with no extramural tumor extension; invasion of contiguous mesenchymal tissue; invasion of adjacent organs or other anatomic structures; involvement of regional lymph nodes; or metastatic spread to distant organs, lymph nodes or other structures[1-3]. CT colonography is available for detecting colorectal masses. Pickhardt *et al*[35] performed CT colonography in 1233 asymptomatic adults referred for CC, and found the sensitivity of virtual colonoscopy for adenomatous polyps was 93.8% for polyps at least 10 mm in diameter, 93.9% for polyps at least 8 mm in diameter, and 88.7% for polyps at least 6 mm in diameter. The specificity of virtual colonoscopy for adenomatous polyps was 96.0% for polyps at least 10 mm in diameter, 92.2% for polyps at least 8 mm in diameter, and 79.6% for polyps at least 6 mm in diameter. CT virtual colonoscopy with the use of a three-dimensional approach is an accurate screening method for the detection of colorectal neoplasia in asymptomatic average-risk adults and compares favorably with optical colonoscopy in terms of the detection of clinically relevant lesions[35].

CT can show diverticulosis and inflammatory bowel disease. CT findings in inflammatory disease include circumferential wall thickening, serpiginous soft-tissue strands extending into the mesenteric fat, and enlarged lymph nodes in the same region[5].

MR colonography and CT were used on 5 patients with inflammatory bowel disease, and 4 of 5 were correctly identified. MR colonography and CT failed to correctly identify in one patient who was diagnosed to have non-specific colitis by histopathologic examination. Currently, colonoscopy is generally reserved for patients with positive results from screening tests or those with a higher than average-risk of colorectal cancer, rather than applying it for routine screening. In the search for an adequate screening method, MR colonography has emerged relatively strongly. MR colonography possesses unique advantages over existing screening tests in that it is quick, less invasive, with no need for sedation or analgesics during investigation and with a lower percentage of perforation complications. Moreover, it enables evaluation of all colon segments because of multi-sectional imaging availability, thus enabling to evaluate intramural, extra-intestinal components of colonic lesions, metastasis and additional lesions. MR colonography is a fundamentally new imaging technique with the potential to alter current clinical approaches in the detection of colorectal neoplasms and

inflammatory bowel disease.

In conclusion, in the search for a rapid, less invasive, accurate, and well-tolerated colorectal examination method, magnetic resonance colonography can be an effective method.

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BASIC RESEARCH

Effect of verapamil on nitric oxide synthase in a portal vein-ligated rat model: Role of prostaglandin

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Abstract

AIM: To investigate the effects of verapamil on nitric oxide (NO) synthesis in a portal vein-ligated rat model.

METHODS: Systemic and splanchnic hemodynamics were measured by radiolabeled microspheres in portal hypertensive rats after acute administration of verapamil (2 mg/kg) on chronic treatment with N^{w}–nitro-L-arginine (NNA)(80 mg/kg) and/or indomethacin (2 mg/kg) .

RESULTS: Verapamil (2 mg/kg) caused a marked fall in both arterial pressure and cardiac output accompanied by an insignificant change in the portal pressure and no change in portal venous inflow. This result suggested that verapamil did not cause a reduction in portal vascular resistance of portal hypertensive rats, which was similar between N^{w}- nitro–L-arginine-treated and indomethacin-treated groups.

CONCLUSION: In portal hypertensive rats pretreated with NNA and/or indomethacin, acute verapamil administration can not reduce the portal pressure, suggesting that NO and prostaglandin play an important role in the pathogenesis of splanchnic arterial vasodilation in portal hypertension.

Key words: Verapamil; Nitric oxide synthase; Portal hypertension

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INTRODUCTION

Verapamil, a calcium entry blocker, in addition to its inhibiting action on the contraction of rat portal vein[1,2], can decrease hepatic venous pressure gradient[3,4] and is proposed as a drug in the treatment of variceal bleeding in patients with HBsAg-positive cirrhosis[4,5]. It was reported that verapamil reduces the intrahepatic shunting in cirrhotic rat- perfused liver[6]. Furthermore, verapamil can also reverse the altered microvascular exchange caused by sinusoidal capillarization in cirrhotic rats when given acutely or chronically[6,7]. However, studies testing verapamil, nifedipine and nicardipine in patients with cirrhosis have failed to show any reduction in hepatic vein pressure gradient, but increase in portal collateral blood flow, an effect that may be dangerous in patients with esophageal varices[8-10]. So far, the mechanism responsible for the effect of verapamil on splanchnic circulation in the rat model of pre-hepatic portal hypertension is still unknown.

Nitric oxide (NO) and prostacyclin (PGI2) are endogenous vasodilators synthesized by the vascular endothelium[11-13]. In fact, both can modulate the mesenteric vascular tone and are important contributors to splanchnic arterial vasodilation in portal hypertensive rats[12,13]. In addition, some investigators have reported that NO plays a major role in modulating collateral vascular resistance[14-16]. Increased PGI2 activities have been observed in the splanchnic vascular bed of portal hypertensive rat model[17-19]. However, it is unknown whether both vasodilators modify the response of splanchnic arterial vasodilation to verapamil in portal hypertensive rats. Thus, we aimed to identify any unique advantageous or deleterious haemodynamic effect of acute verapamil on nitric oxide synthese in a pre-hepatic portal hypertensive rat model. In addition, the influence of NO and prostaglandin on the responsiveness of splanchnic arterial vasodilation to verapamil was also evaluated.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 280-340 g at the time of surgery were used for experiments. The rats were housed in a plastic cage and allowed free access to food and water. All rats were fasted for 12 h before operation. In all experiments, the investigators followed the American Physiological Society Guiding Principles for the Care and Use of Laboratory Animals.

A prehepatic portal hypertensive animal model was induced by partial portal vein ligation (PVL) as previously described[20]. Anesthesia was performed with ketamine HCl (100 mg/kg body weight, intramuscularly). In brief, the portal vein was isolated and a 3-0 silk ligature was tied around the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed and the vein allowed to reexpand. A second loose ligature was left around the portal vein with the 2 ends of the ligature placed on each side in the abdominal cavity. The abdomen was then closed and the animal was allowed to recover. Perfusion studies were performed on overnight-fasted rats 10-13 d after the operation, at which time an extensive collateralization of the portal system was fully established.

Experimental design

Rats with a ligated portal vein were divided into three groups. In each group of PVL rats, verapamil (Isoptin Knoll AG, Ludwigshafen, Germany) was given at a dose of 2 mg/kg parenterally, which decreased the mean arterial pressure (MAP) by more than 10%. One group of sham-operated rats received sterile saline (1 mL/kg) only. A group of PVL rats were also studied after the administration of sterile saline (1 mL/kg) alone to ensure that the hyperdynamic state could be achieved. Pressure dose-response curves were constructed for each group of rats 15 min after the administration of each dose. Seven doses were used at the concentration ranging from 0.02 to 2.0 mg/kg body weight. Verapamil was administered through a venous catheter. In the first group, sham-operated and PVL rats were treated daily following the ligation with one ip injection of N^{w}–nitro-L-arginine (NNA) (80 mg/kg). In the second group, sham-operated and PVL rats were treated daily following the ligation with one ip injection of indomethacin (INDO) (2 mg/kg). In the third group, sham-operated and PVL rats were treated daily following the ligation with ip injection of both NNA (80 mg/kg) and indomethacin (2 mg/kg).

Experiment preparation

All animals were anaesthetized with pentobarbital (50 mg/kg) and fastened on an animal board in dorsal recumbency. Both femoral vein and artery were cannulated with a polyethylene catheter (PE-50). The mean arterial blood pressure (MAP) was measured with a strain-gauge transducer (Statham P23 Db) connected to the femoral artery cannula and recorded continuously on a Grass polygraph (Grass Apparatus, Quincy, MA, USA). The skin over the left upper abdominal quandrant was shaved, a small (1.0 cm-2.0 cm) parasagittal incision was made at the level of the left anterior axillary line and the muscles were gently separated with forceps. The spleen was exposed by pulling it away from the perisplenic fat and then covered with a piece of warm Ringer-lactate moist gauze. Special care was taken to avoid any unnecessary manipulation of the spleen. The jejunal vein was cannulated with a PE-50 catheter for the continuous monitoring of portal pressure. Catheters were also placed in the left femoral artery and right carotid artery. The right carotid artery catheter was carefully advanced into the left ventricle under continuous pressure. Heart rate measurement was used to radiolabel the microsphere injection for the determination of portal venous inflow (PVI). All catheters were connected to Statham P-23-Db strain gauge transducers (Statham), and continuous pressure recordings of left ventricular and portal pressures (PP) were printed on a Grass model ID inscription recorder (Grass, Quincy, MA, USA). Baseline pressure and heart rate measurement were recorded in each animal.

Systemic and splanchnic hemodynamics

Splanchnic organ blood flow and cardiac output were determined twice (before and 15 min after administration of the drug) according to the reference sample method with intracardiac injection of isotope-labelled microspheres (15±3 μm in diameter)[21]. 「^{57}Co」 and 「^{113}Sn」-labelled microspheres (New England Nuclear, Boston, MA, USA) suspended in Tween 80 (0.05%) were used for the first or second injection. Approximately 180 000 「^{57}Co」 and 「^{113}Sn」-labelled microspheres were aspirated into plastic 1.0 mL syringes (volume 0.3-0.4 mL) and mixed in a vortex for 5 min. An additional 0.2 mL sample containing approximately 30 000 「^{46}Sc」-labelled microspheres was also placed into a 1.0 mL plastic syringe until injection.

Ten seconds after withdrawal of a blood sample from the left femoral artery for reference, microspheres were injected into the left ventricle over 10-15 s. Catheters were flushed with 0.2 Ml of 0.9% NaCl. Blood was withdrawn from the left femoral artery over 90 s at an approximate rate of 1 mL/min using a Harvard pump (Harvard Apparatus, Millis, MA, USA). Once withdrawal was complete, a volume of 0.9% NaC1 equal to the sample volume was withdrawn and reinfused, arterial blood pressure was monitored to ensure stability during microsphere distribution. As soon as the spleen was exposed, the 「^{46}Sc」-labelled microspheres were injected through a 23-gauge needle into the splenic pulp over a period of 20 s. The animals were then killed with an injection of bolus of saturated KC1 into the carotid artery catheter.

The liver, lungs, stomach, intestine, spleen, pancreas, mesentery and kidneys were dissected and weighed. The radioactivity of the organs was determined in a scintillation counter (Packard, Downers Grove, IL, USA) with an energy window set at 50-200 KeV for ^{57}Co, at 300-500 KeV for ^{113}Sn and at 800-1200 KeV for ^{46}Sc, respectively. At least 300 microspheres were trapped in the reference sample and organs to ensure validity of the measurement. The error in the measurement of radioactivity induced by spillover of each radioactive microsphere channel was corrected using ^{57}Co, ^{113}Sn and ^{46}Sc standards. Each standard was checked by a multichannel analyzer (Series

Table 1 Baseline characteristics in different study groups (mean ± SD)

Groups	*n*	Body weight (g)	Mean arterial pressure(mmHg)	Heart rate (beat/min)
Vehicle	8	326.7±10.6	98.6±3.6	296±18
Verapamil	8	332.6±11.8	96.5±4.8	320±16
Verapamil+vehicle	8	321.5±9.8	99.5±5.2	318±20
Verapamil+NNA	8	330.8±9.6	97.4±3.8	317±17
Verapamil+INDO	7	327.8±8.5	94.2±6.2	328±14
Verapamil+INDO+NNA	8	319.8±7.9	96.2±4.8	308±15

NNA: N^{ω}-nitro-L-arginine; INDO: indomethacin.

35 PLUS). Adequate microsphere mixing was assumed at a difference < 20% between the left and right kidneys. Any animal not meeting this requirement was rejected from the analysis. Cardiac output was calculated according to the following formula:

Cardiac output (mL/min) = (injected radioactivity (cpm) × reference sample blood flow (mL/min)) / reference sample radioactivity (cpm).

Injected radioactivity was calculated on the basis of the difference between the initial and residual radioactivity in the syringe. Organ blood flow was calculated as follows:

Organ blood flow (mL/min) = (organ radioactivity (cpm) × reference sample blood flow (mL/min)) / reference sample radioactivity (cpm).

Vascular resistance of different organs in the portal venous system was calculated according to the following formula:

Resistance (dyn.s.cm^{-5}×10^5) = (mean arterial pressure (mmHg) - portal venous pressure (mmHg)) × 80/organ blood flow (mL/min)

Portal tributary blood flow was calculated as the sum of the blood flow in the spleen, stomach, colon, mesentery and pancreas. The hepatic arterial blood flow was taken to be equal to the liver blood flow.

Portal systemic shunt (PSS) % was calculated as:

[ct/min in lung/ct/min in (lung + liver)] x 100

Drugs

Verapamil, NNA, INDO, and reagents for preparing Krebs' solution were purchased from Sigma. All solutions were freshly prepared on the day the experiment was conducted.

Statistical analysis

Results were expressed as mean ± SD. The concentration of verapamil at 50% of the maximal response (EC_{50}) in each preparation was calculated from sigmoid logistic curves and expressed as negative log molar (-log mol/L). Statistical analyses were performed by the paired or unpaired Student's *t* test and one-way analysis of variance with Tukey's test when appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Systemic and splanchnic hemodynamics

No differences were observed in body weights, mean arterial pressures and heart rates among the three groups (Table 1). The PVL rats exhibited a hyperdynamic state of splanchnic circulation with a high cardiac index. The pressure-dose-response curves of the sham-operated and PVL rats after acute verapamil administration in different groups were similar to the curves previously reported by us[20](Figure 1).

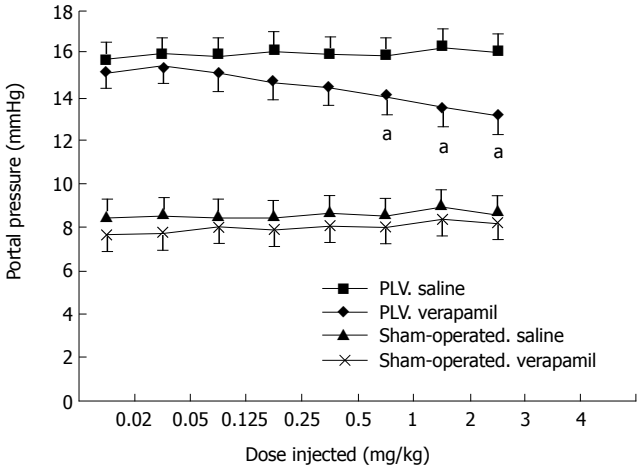

Figure 1 Portal pressure responses to verapamil in four groups of rats: $^aP<0.05$ *vs* sham-operated control. PVL: portal vein ligated.

No significant change of the portal pressure (PP) was noted when verapamil (2 mg/kg) was given to PVL rats by early chronic administration of NNA, INDO, or both in all three groups. The results of this study also indicated that acute verapamil administration could reduce the PP in different study groups of PVL rats in a hyperdynamic state. In all three groups of rats, the mean arterial pressure (MAP) significantly decreased following administration of verapamil compared to the control group. In contrast, the portal pressure, portal venous inflow (PVI) and hepatic arterial flow (HAF) were maintained, while cardiac output (CO) decreased significantly compared to the control group (Table 2, Figure 2). Thus, the effect of verapamil on chronic treatment with NNA (80 mg/kg), INDO (2 mg/kg), and both on splanchnic organ blood flow in sham-operated and PVL rats after 15 d from the control are shown in Figure 3. At a dose of 2 mg/mL verapamil, there was also no significant decrease in total peripheral resistance (TPR), splanchnic arterial resistance (SAR) and portal venous resistance (PVR) in all three groups of PVL rats (Table 3).

Portal-systemic shunting

After PVL rats were pretreated with either NNA or indomethacin for 15 d, acute verapamil (2 mg/kg) administration did not induce any significant reduction in the portal-systemic shunting. Furthermore, the portal pressure in both NNA- and indomethacin-pretreated PVL rats was also not significantly different from that in the control group. The portal pressure reduction was not correlated with portal-systemic shunting reduction in either NNA- or indomethacin-pretreated PVL rats (r = 0.05, NS; r = 0.03, NS).

Table 2 Effect of verapamil in combination with NNA, INDO, and NNA plus INDO on systemic and splanchnic haemodynamics in PVL rats (mean ± SE)

	Verapamil (n = 8)		Verapamil + NNA (n = 8)		Verapamil + INDO (n = 7)		Verapamil + NNA + INDO (n = 8)	
	Before	After	Before	After	Before	After	Before	After
CI[mL/(min.kg)]	423±38	298±42[a]	425±41	312±48[a]	424±39	332±51[a]	426±40	326±49[a]
MAP(mmHg)	105±5	96±6[a]	106±6	99±7[a]	107±4	98±7[a]	106±8	100±7[a]
PP(mmHg)	15.0±1.6	10.8±1.9[a]	14.3±1.8	13.8±2.1	15.2±1.9	14.5±2.4	15.1±1.6	14.6±1.4
PVI(mL/min)	27±2.8	26±3.2	26±2.7	25±3.4	28±3.1	27±3.5	27±3.2	26±3.9
HAF(mL/min)	7±1.6	8±1.8	8±1.8	9±2.2	7±1.9	8±2.2	9±2.0	9±2.8
PSS(%)	81.7±7.2	63.6±8.2[a]	80.2±8.3	76.5±9.2	81.2±6.2	77±7.8	80.5±6.6	76.2±9.5

CI: cardiac index; MAP: mean arterial pressure; PP: portal pressure; HAF: hepatic arterial flow; PSS:portalsystemic shunting; NNA: N^{w}-nitro-L-arginine; INDO: indomethacin; [a]$P < 0.05$ *vs* control group.

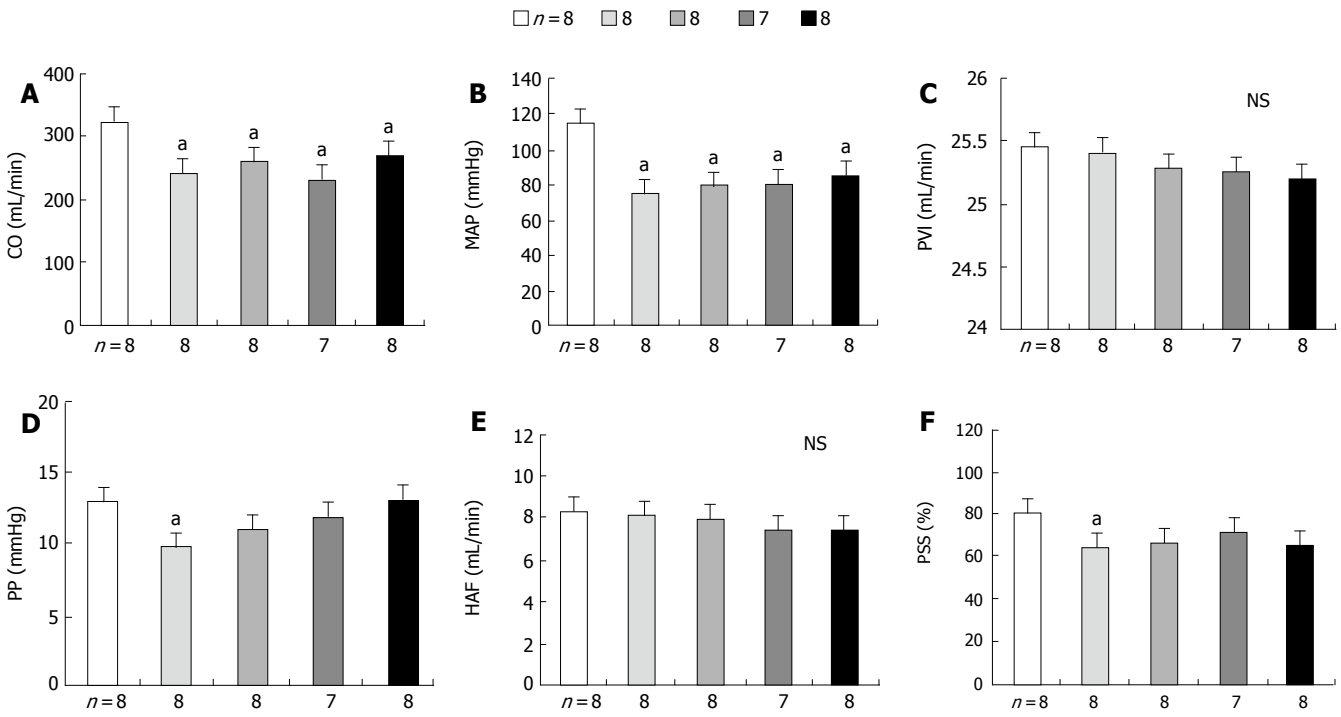

Figure 2 Effect of verapamil in combination with NNA (□ light grey), INDO (■ dark grey) and NNA plus INDO (■ black) on cardiac output (**A**), mean arterial pressure (**B**), portal venous inflow (**C**), portal pressure (**D**), hepatic arterial flow (**E**), and portal systemic shunting (**F**) in PVL rats. [a]$P < 0.05$ *vs* control group.

Table 3 Effect of verapamil in combination with NNA, INDO, and NNA plus INDO on systemic and splanchnic vascular resistance in PVL rats (mean ± SE)

	Verapamil (n = 8)		Verapamil + NNA (n = 8)		Verapamil + INDO (n = 7)		Verapamil + NNA + INDO (n = 8)	
	Before	After	Before	After	Before	After	Before	After
TPR (dyh.s.cm^{-5}×10^{4})	8.96±0.87	6.45±0.78[a]	8.84±0.72	8.47±0.98	8.76±0.88	8.52±0.91	8.78±0.93	8.58±0.89
SAR (dyh.s.cm^{-5}×10^{5})	4.38±0.72	3.16±0.62[a]	4.51±0.89	4.18±0.71	4.45±0.92	4.20±0.84	4.46±0.84	4.22±0.94
PVR (dyh.s.cm^{-5}×10^{4})	7.49±0.68	6.12±0.58[a]	7.46±0.72	7.23±0.67	7.54±0.97	7.41±0.95	7.46±0.65	6.95±0.87

TPR: total peripheral resistance; SAR: splanchnic arterial resistance; PVR: portal venous resistance; NNA: N^{w}-nitro-L-arginine; INDO: indomethacin. [a]$P < 0.05$ *vs* control group.

DISCUSSION

The results of the present study showed that verapamil administration did not favorably influence splanchnic arterial vasodilation in portal vein-ligated rats pretreated with NNA and indomethacin as expected.

These findings are not consistent with those of a previous study in rats with carbon tetrachloride-induced cirrhosis[6], in which rat model it was demonstrated that verapamil reduces hepatic resistance, exerts beneficial effect on the hepatic microvascular exchange, improves liver function and reduces portal pressure if chronically administered. It was reported that verapamil is a useful therapeutic agent for patients with cirrhosis[4]. In a small group of patients, Freeman *et al*[3] demonstrated that verapamil administered intravenously, decreases the hepatic venous pressure gradient (HVPG). Furthermore, we

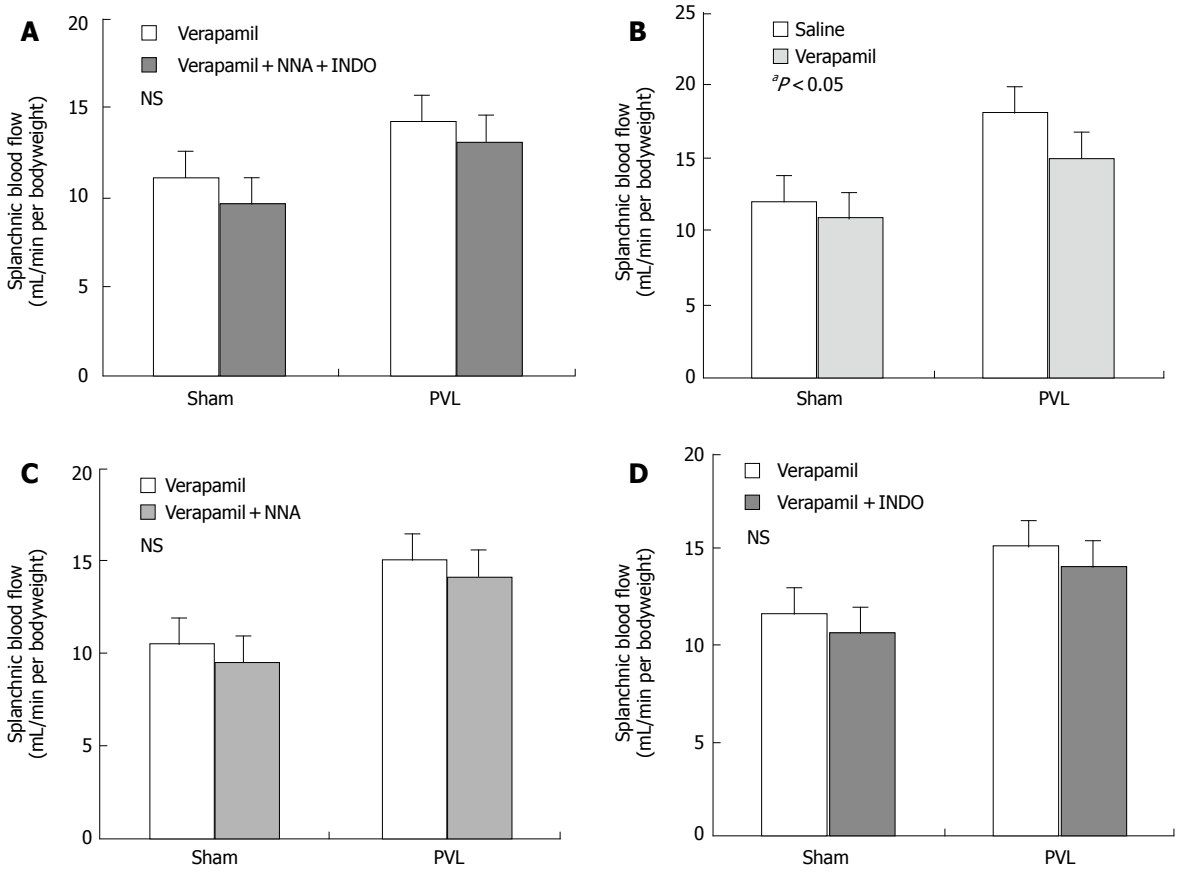

Figure 3 Effects of verapamil in combination with NNA (80 mg/Kg), INDO (2 mg/Kg) and NNA plus INDO on splanchnic blood flow in sham-operated and PVL rats after 15 days. **A**: verapamil plus NNA plus INDO; **B**: verapamil alone; **C**: verapamil plus NNA; **D**: verapamil plus INDO. $^aP < 0.05$ *vs* control group.

have obtained similar results in patients with postnecrotic cirrhosis after both acute and chronic administration of verapamil[4,5].

The present study failed to document any beneficial effect of verapamil in portal hypertensive rats pretreated with NNA and indomethacin. This might not be due to a low dose or a poor absorption of verapamil, as documented by the dose response curve which was well within the therapeutic range. Pharmacodynamic efficacy was also manifested by the observed systemic effects of verapamil, including increased HR and peripheral vasodilation as well as decreased arterial pressure and SVR. Verapamil did not exert any noticeable effect on portal pressure, PVI and HAF. Accordingly, the hepatic vascular resistance did not decrease in our study. Because of this lack of effect on the splanchnic and hepatic arterial vasodilation, verapamil failed to influence liver perfusion. Thus, our results do not support the use of verapamil in the treatment of cirrhosis and portal hypertension pretreated with NNA and indomethacin. Further evidence against the use of verapamil in portal hypertension has been demonstrated by Narasa *et al*[8] who in accordance with our findings have failed to document any decrease in portal pressure following verapamil administration in portal hypertensive patients.

The reason for the discrepancies between the current results and previous studies is not clear. It may be related to the fact that our rat model was produced by portal vein ligation, causing presinusoidal portal hypertension, and these rats were pretreated with NNA and indomethacin. It is also possible that in this kind of animal model, verapamil cannot reduce portal pressure by decreasing intrahepatic portal resistance, and improve hepatic function by affecting the apparent hepatic blood flow[22]. So far, the effect of verapamil on rat hepatic stellate cells is still unknown[23]. The structural changes have not been observed and within the liver the hepatic microcirculation cannot be pharmacologically altered because verapamil has a high first-pass effect[1,24]. However, we have previously documented in a similar model of portal hypertensive rats that the hepatic vascular resistance and portal pressure can be lowered by verapamil[4]. Although animal experiments have demonstrated that verapamil can produce a complex interplay of alterations in preload (splanchnic venodilatation), afterload and myocardial contractility by inhibiting the constrictor responses of the splanchnic capacitance vessels of small resistant arterioles and arteries to the sympathetic nervous outflow[1,6,7], this finding indicates that the effect of verapamil on splanchnic arterial vasodilation can be pharmacologically modified by pretreatment with NNA and indomethacin.

Nitric oxide and prostaglandin are endogenous vasodilators produced by vascular endothelial cells[11-15]. Non-specific inhibition of NO synthesis can restore mesenteric vascular responsiveness[25] and normalize splanchnic vascular resistance[26], thus ameliorating portal-systemic shunting and hyperdynamic circulation[27,28]. Both NO and prostaglandin participate in the regulation of

vascular tone in different vascular beds of normal and portal hypertensive animals[15-18]. It has been shown that in portal hypertensive rats, inhibition of nitric oxide synthesis increases not only intrahepatic and splanchnic vascular resistance but also portal-collateral resistance[11,15,29]. Recently, increased prostacyclin (PGI 2) activities have been observed in systemic circulation and portal vein segments of portal hypertensive rats[18]. In addition, prostaglandin is involved in the modulation of collateral vascular tone in portal hypertensive rats and can modify the vasoconstrictive effect of vasopressin[25]. Nitric oxide and PGI 2 produce relaxation of the vascular smooth muscle by different mechanisms. Nitric oxide works by activating guanylate cyclase which increases the intracellular levels of guanosine 3, 5-cyclic monophosphate[12].

In conclusion, acute verapamil administration does not increase the splanchnic arterial vasodilation in pre-hepatic portal hypertensive rats pretreated with NNA and /or indomethacin. In conjunction with our previous reports[20], these findings indicate that both NO and prostaglandin can modulate the splanchnic vascular response to verapamil and may therefore participate in the development and maintenance of portal hypertension.

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BASIC RESEARCH

Relationship between transforming growth factor β1 and anti-fibrotic effect of interleukin-10

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Abstract

AIM: To study the effect of interleukin-10 (IL-10) on the expression of transforming growth factor β1 (TGF-β1) in hepatic fibrosis rats and the anti-fibrotic role of exogenous IL-10.

METHODS: Hepatic fibrosis was induced by carbon tetrachloride administered (CCl_4) intraperitoneally. The experiment was performed in two stages. In the first stage, 60 SD rats were divided randomly into normal control group 1 (GN_1, n = 8), hepatic fibrosis group (GC, n = 28) and IL-10 intervened group (GI, n = 24). At the beginning of the 7th and 11th wk, hepatic stellate cells (HSCs) were isolated, reverse transcription-polymerase chain reation (RT-PCR) and immunocytochemistry were performed to detect the expression of TGF-β1 in HSCs. Histological examination was used to determine the degree of hepatic fibrosis. In the second stage, 47 SD rats were divided randomly into normal control group 2 (GN_2, n = 6) and CCl_4 group (GZ, n = 41). At the end of the 9th week, rats in GZ group were allocated randomly into model group (GM, n = 9), IL-10 treatment group (GT, n = 9) and recovered group (GR, n = 9). At the end of the 12th week, all rats were sacrificed. RT-PCR and immunohistochemistry were performed to detect the expression of TGF-β1 in liver tissue. ELISA was used to assay serum TGF-β1 levels.

RESULTS: Hepatic fibrosis developed in rats with the increase of the injection frequency of CCl_4. In the first stage, hepatic fibrosis developed and HSCs were isolated successfully. At the 7th and 11th week, TGF-β1 mRNA in GC group increased significantly compared with that in GN_1 (P = 0.001/0.042) and GI groups (P = 0.001/0.007), whereas there was no significant difference between the two groups. The levels of TGF-β1 at the beginning of the 7th wk was higher than that of the 11th wk (P = 0.049). Immunocytochemistry results of TGF-β1 were consistent with the above findings. In the second stage, TGF-β1 increased significantly in GM group compared to GN_2. After treatment with IL-10, TGF-β1 declined obviously. The expression of TGF-β1 decreased in GR group but was still higher than that in GT group.

CONCLUSION: The levels of TGF-β1 are increased in hepatic fibrosis rats and decreased after treatment with exogenous IL-10. IL-10 may play an anti-fibrotic role by suppressing TGF-β1 expression.

Key words: Hepatic fibrosis; Hepatic stellate cells; Interleukin-10; Transforming growth factor-β1

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INTRODUCTION

Hepatic fibrosis, which represents the wound healing response of the liver, is a common sequel of liver injury characterized by increased deposition and altered composition of extracellular matrix (ECM) [1-2]. Hepatic stellate cells (HSCs) are the major source of ECM and regarded as the principle cell type in the development of hepatic fibrosis [3-5]. TGF-β1 appears to be the main fibrogenic mediator and exerts its effects through autocrine or paracrine on HSCs [6]. Hepatocytes also express TGF-β1 when isolated and cultured *in vitro* as a response to an altered extracellular environment. It has been reported that interleukin-10 (IL-10) could relieve the degree of rat hepatic fibrosis induced by carbon tetrachloride (CCl_4) [7]. In the present study, we isolated HSCs and detected the expression of TGF-β1 in HSCs and liver tissues, and explored the relationship between TGF-β1 and hepatic fibrosis in an attempt to explain the possible mechanisms of the antifibrotic activities of exogenous IL-10 *in vivo*.

MATERIALS AND METHODS

Establishment of models

All rats were bred under routine conditions (at room temperature of 22 ℃ ± 2 ℃, a humidity of 55% ± 5%) with a light/dark cycle and free access to drinking water and food (BK Company in Shanghai, China). In the first stage, 60 clean male SD rats were divided randomly into normal control group 1 (GN_1, n = 8), hepatic fibrosis group (GC, n = 28) and IL-10 intervened group (GI, n = 24). Rats in GN_1 group were injected intraperitoneally with saline at a dose of 2 mL/kg twice a week. Rats in the other two groups received intraperitoneal injection of 50% CCl_4 dissolved in castor oil (2 mL/kg) twice a week as described previously[8]. From the third week, rats in GI group were injected intraperitoneally with IL-10 dissolved in saline (4 μg/kg) 20 min before CCl_4 administration as previously described[9]. The intervention lasted to the end of the experiment. In the second stage, 47 SD rats were divided randomly into normal control group 2 (GN_2, n = 6) and CCl_4 group (GZ, n = 41). Rats in the GN_2 group were injected intraperitoneally with saline at a dose of 2 mL/kg, twice a week. Rats in the GZ group received intraperitoneal injection of 50% CCl_4 dissolved in castor oil (2 mL/kg) twice a week. At the end of the 9th week, rats in the GZ group were divided randomly into model group (GM, n = 9), IL-10 treatment group (GT, n = 9) and recovered group (GR, n = 9). Rats in the GM group were injected with CCl_4 continuously: Rats in the GT group received intraperitoneal IL-10 dissolved in saline (4 μg /kg) after injection of CCl_4 for 9 weeks and rats in the GR group were not treated. Three rats in the GN_2 group were executed at the 9th week and the other rats were sacrificed at the 12th week.

Histological examination

In the first stage, at the beginning of the 7th and 11th week, 2 rats from each group were chosen randomly for histological examination. Liver tissues were fixed in 10% formalin and embedded with paraffin. Sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

Isolation, incubation and identification of HSCs

Isolation, incubation and identification of HSCs were performed as previously described[10]. Briefly, at the beginning of the 7th and 11th week, 5 rats from each group were chosen randomly to perfuse with 0.13% pronase E and 0.025% type-IV collagenase through a portal vein catheter. The liver tissue suspension was incubated with 0.02% pronase E and 0.025% type-IV collagenase under agitation. The suspension obtained from the digested liver tissue was centrifuged with 11% Nycodenz density gradient to purify HSCs. Then, the HSCs were seeded at the concentration of 1×10^6 cells /mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum in 96-well plates and kept at 37 ℃ in a 50 mL/L CO_2 atmosphere for 72 h. The HSCs were identified by their typical phase-contrast microscopic appearance and immunocytochemistry using antibody directed against desmin. Cell vitality was checked by trypan-blue exclusion.

RNA extraction and RT-PCR

Total RNA was extracted from freshly isolated HSCs and liver tissues according to the instructions of the RNA isolation kit (Jingmei Biotechnology Company, Shenzhen). Its quantity and purity were assessed by measuring the optical density at 260 nm and 280 nm. After measurement of RNA amount, samples were either used immediately for RT or stored at -70 ℃.

For RT, 1 μg total RNA was transcribed following the instructions of the first strand cDNA synthesis kit (Jingmei Biotechnology Company, Shenzhen). Twenty μL reaction mixture was transcribed at 42 ℃ for 60 min, at 99 ℃ for 5 min and stored at -20 ℃.

For PCR, primers coding for the house-keeping gene-β-actin were added into the reaction mixture to standardize the results. Fifty μL aqua was added into the reactive system containing 2 μL cDNA, 5 μL 10×buffer, 5 μL 25 mmoL/L $MgCl_2$, 1 μL 10 mmoL/L dNTP, 1 μL 20 mmoL/L up stream and down stream primer of target gene, 1 μL 20 mmoL/L β-actin primer pairs, 3U Taq DNA polymerase. Then PCR was performed with predenaturation at 94 ℃ for 5 min followed by 30 cycles at 94 ℃ for 45 s, at 55 ℃ for 30 s, at 72 ℃ for 60 s and a final extension at 72 ℃ for 7 min. Primers were designed according to the reference of GenBank (Table 1). PCR products were immediately analyzed by 20 g/L agarose gel electrophoresis and the density of resultant bands was semi-quantified by scanning densitometry using the ratio of TGF-β1 to β-actin to assess the relative level.

Table 1 Primer sequences used in the study

mRNA	Upstream primer	Downstream primer	Product length (bp)
TGF-β1	CTC TGC AGG CGC AGC TCT G	GGA CTC TCC ACC TGC AAG AC	392
β-actin	CCA ACC GTG AAA AGA TGA CC	CAG GAG GAG CAA TGA TCT TG	660

Immunocytochemistry

Most of HSCs attached to the dishes after 72 h of primary culture. The 96-well plates were washed twice with 0.1 moL/L PBS and cells were fixed with poly-formaldehyde at 4 ℃ overnight. The following procedures were performed according to the instructions of streptavidin/peroxidase (S-P) kit (Beijing Zhongshan Company). Briefly, cells were washed with PBS, incubated with bovine serum albumin in PBS, reacted with primary antibody dissolved in PBS, washed, incubated with peroxidase-conjugated second antibody, washed again and reacted with S-P for 20 min. Color reaction was developed by incubation with DAB. In negative controls, the primary antibody was replaced by PBS.

Immunohistochemistry of hepatic TGF-β1

After dewaxed with xylene and rehydrated through a graded alcohol series, sections were digested with 4 g/L trypsin. The following procedures were similar to the steps of immunocytochemistry. Slides were then analyzed with an image analyzing system to obtain the relative contents of hepatic TGF-β1. The characteristics of the antibodies used in this study are summarized in Table 2.

Table 2 Antibodies used for immunocytochemistry and immunohistochemistry

Antibody	Type	Source	Working solution
Desmin	Polyclonal, mouse	Beijing Zhongshan Co.	1:100
TGF-β1	Polyclonal, rabbit	BOSTER Co.	1:20/1:25
Antibody to rabbit IgG	Goat	Beijing Zhongshan Co.	Ready to use
Antibody to mouse IgG	Goat	Beijing Zhongshan Co.	Ready to use

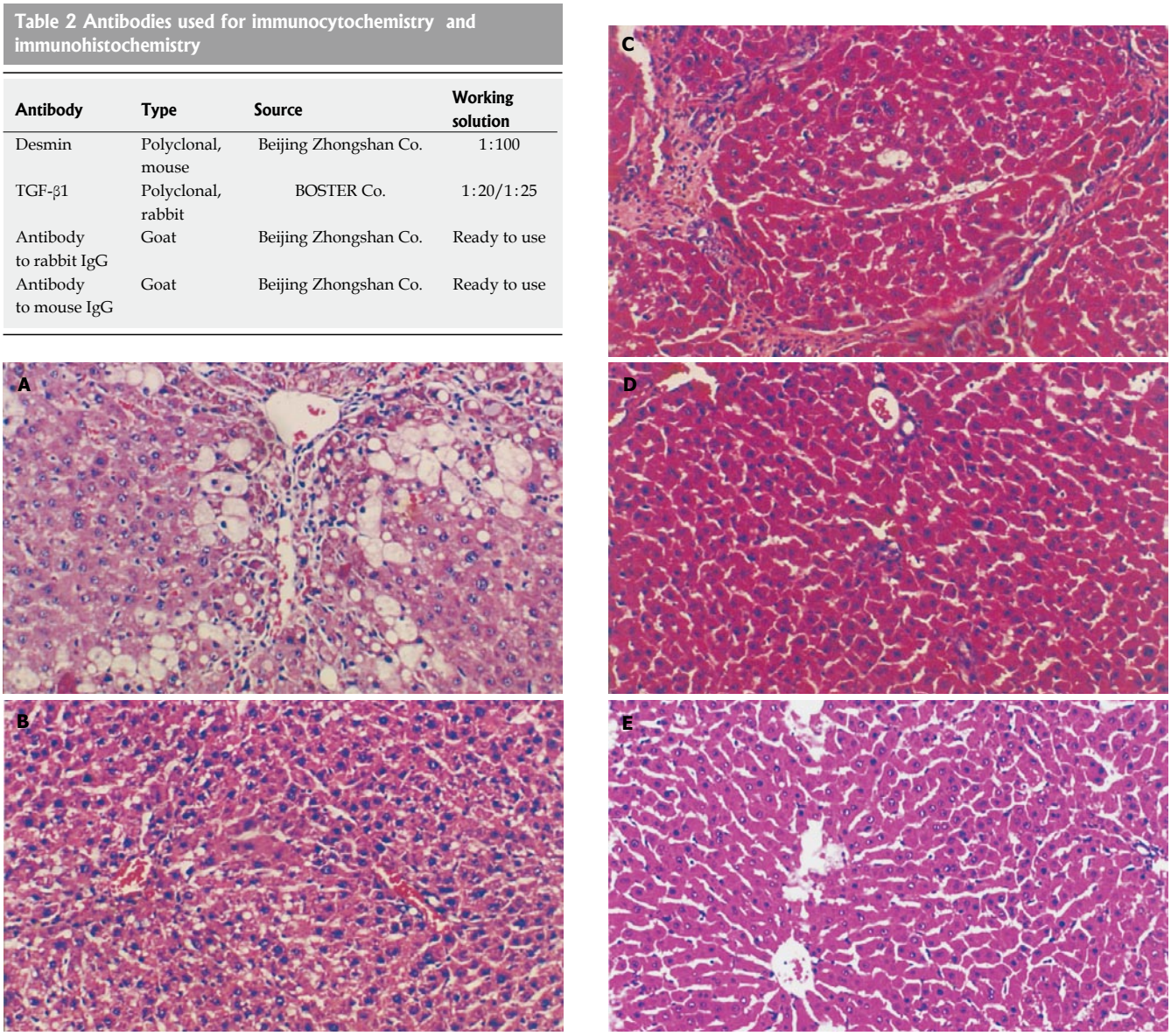

Figure 1 Effect of IL-10 on liver histological change in group C at the 7th wk (**A**), group I at the 7th wk (**B**), group C at the 11th wk (**C**), group I at the 11th wk (**D**), and group N (**E**).

ELISA of serum TGF-β1

Assay of serum TGF-β1 content was performed with double antibody ABC-ELISA method according to the ELISA kit instructions (Maoyuan Science Technology Limited Company, Shanghai).

Statistical analysis

All data were expressed as mean±SE. The significance for the difference between the groups was studied with SPSS11.0 by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Histological examination

Hepatic fibrosis became remarkable after the treatment with CCl_4. At the 7th wk, specimens from the GC group showed steatosis and ballooning degeneration, the collagen fibers increased and began to extend to the parenchyma, many mononuclear cells and unusual neutrophils surrounded the centrilobular veins and fibrotic septa (Figure 1A), while only a few inflammatory cells infiltrated around the centrilobular veins without evident changes of lobular structure in the GI group (Figure 1B). At the 11th week, the reticular fiber extended into the hepatic plate and full delimitation was developed (Figure 1C), while less fibrosis septa and inflammatory infiltration were seen in the GI group (Figure 1D). Specimens from the GN_1 group showed normal lobular structure (Figure 1E). Due to the limit of samples, no statistical data presented disparity between the two groups, but fibrogenesis in the GI group was much less severe than that in the GC group.

Isolation and identification of HSCs

A total of $2–4.5\times10^7$ cells were harvested from each rat. HSCs were identified by immunoreaction with desmin (Figure 2). The mean purity of freshly isolated HSCs was 95% ± 5%. The cell vitality checked by trypan-

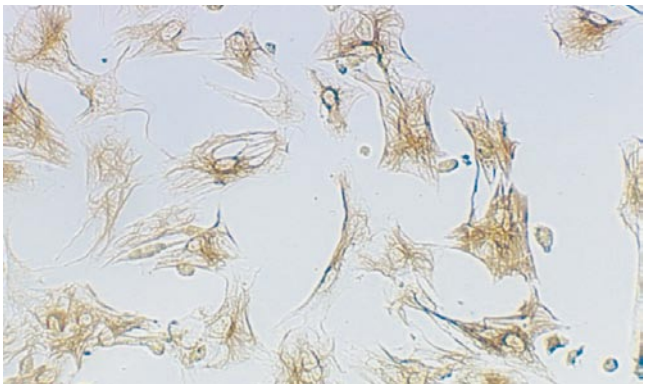

Figure 2 Desmin expression in freshly isolated HSCs (SP × 100).

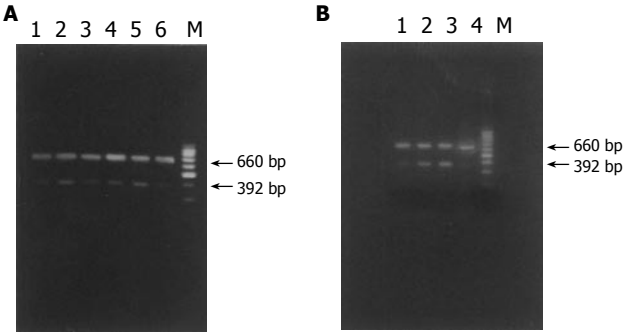

Figure 3 Expression of TGF-β1 in HSCs the first (**A**) and the second stage (**B**).

blue exclusion was higher than 95%.

Expression of mRNA

The purity of RNA was determined by the ratio of OD260 and OD280 ranging between 1.8-2.0. In the first stage, at the 7th and 11th wk, TGF-β1 mRNA in the GC group increased obviously compared to the GN_1 and GI groups ($P < 0.01$), but no difference was seen between the two groups. TGF-β1 expression at the 7th wk was higher than that at the 11th wk ($P < 0.05$, Table 3, Figure 3A). In the second stage, TGF-β1 mRNA in the GM group increased notably compared to the GN_2 group ($P < 0.01$) and declined obviously after treatment with IL-10 ($P < 0.01$). Its expression in the GR group was lower than that in the GM group ($P < 0.01$) but still higher than that in the GT group ($P < 0.05$, Table 4, Figure 3B).

Immunocytochemistry and immunohistochemistry

In the first stage, TGF-β1 positive expressions were localized in cytoplasm and nuclei of HSCs in all groups by immunocytochemistry after 72 h of primary culture. Most of the cells attached to and spread over the plastic substratum. At the 7th wk, the size of HSCs in the GC and GI groups was a little larger than that in the GN_1 group. At the 11th wk, cell phenotype in the GI group showed circle or ellipse and was a little smaller than that in the 7th wk, no obvious change of phenotype was seen in the GC group. Although the number of samples was limited, the expression of TGF-β1 in the GC group was higher than that in the GN_1 group and decreased after treatment with IL-10 (Figures 4A-4D). In the second stage, TGF-β1 positive expressions in the GM group were localized in most

Table 3 Expression of TGF-β1 mRNA in HSCs (mean ± SE)

Wk	*n*	Group N	Group C	Group I
7	5	0.143±0.009	0.267±0.025[b]	0.140±0.008[d]
11	5	0.141±0.004	0.207±0.029[ab]	0.123±0.009[d]

[b]$P<0.01$ *vs* group N; [d]$P<0.01$ *vs* group C; [a]$P<0.05$ *vs* wk 7.

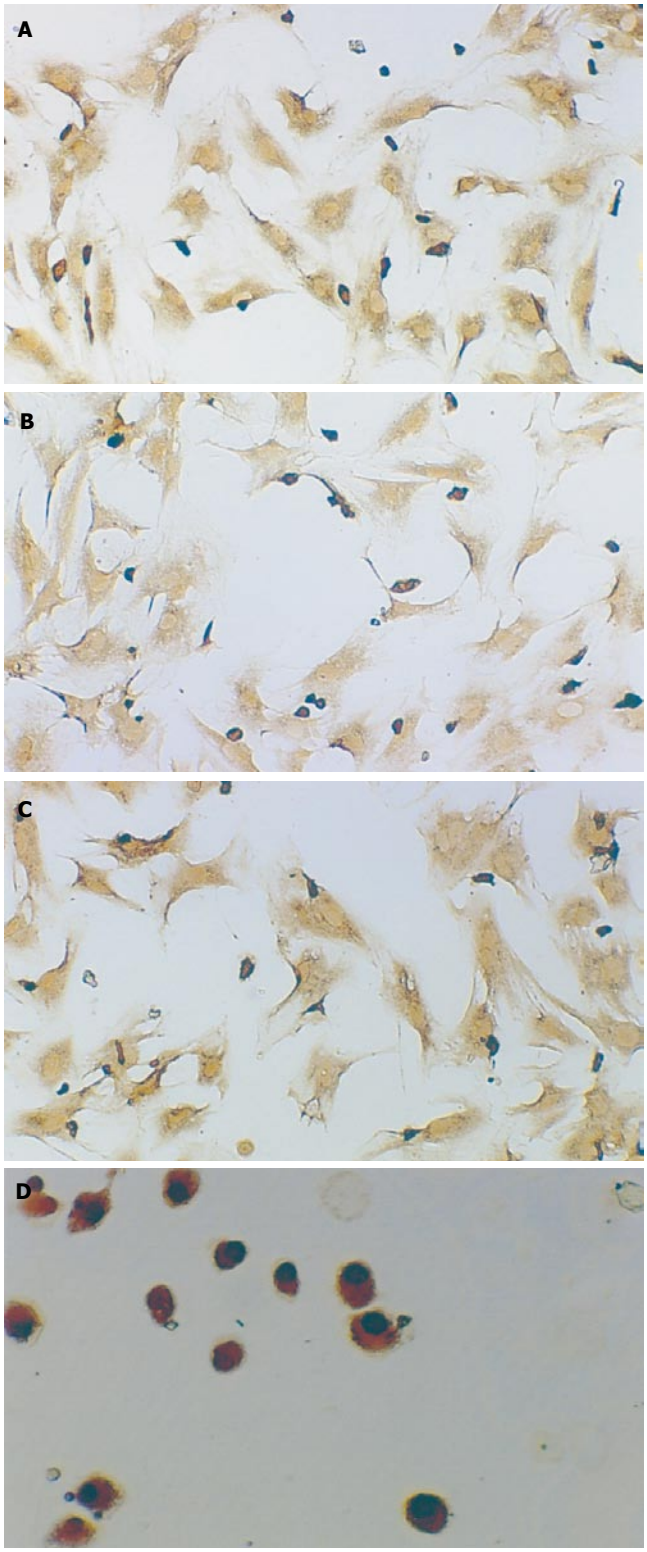

Figure 4 Expression of TGF-β1 protein in HSCs in group C (**A**) at the 7th wk, group I (**B**) at the 7th wk, group C at the 11th wk (**C**), and group I (**D**) at the 11th wk.

Table 4 Expression of TGF-β1 in liver tissues (mean ± SE)

GROUP	*n*	TGF-β1 content		
		Immunhistochemistry	RT-PCR	ELISA
N_2	6	0.810 ± 0.141	0.188 ± 0.014	95.442 ± 35.341
M	9	1.804 ± 0.382[a]	0.449 ± 0.040[b]	242.593 ± 53.902[a]
R	9	0.963 ± 0.121[c]	0.279 ± 0.015[bd]	165.514 ± 23.193[ac]
T	9	0.501 ± 0.250[ace]	0.182 ± 0.027[de]	113.472 ± 15.101[ce]

[a]*P* <0.05, [b]*P* <0.01 *vs* group N; [c]*P* <0.05,[d]*P* <0.01 *vs* group M; [e]*P* < 0.05 *vs* group R.

of the liver tissues, appearing brown. After treatment with IL-10, the distributing area was smaller and the color was lighter. TGF-β1 expression in the GR group was lower than that in the GM group but still higher than that in the GT group (Table 4, Figures 5A-5D).

Expression of serum TGF-β1

The expression of serum TGF-β1 detected by ELISA in different groups had the same tendency as that detected by RT-PCR and immunohistochemistry in the second stage (Table 4).

DISCUSSION

Although significant progress has been made in understanding the pathogenesis of hepatic fibrosis, a rational therapy that prevents the progression or even reverses fibrosis remains elusive[11-13]. IL-10 is produced mainly by TH_2 cells and inhibits the functions of TH_1 cells. It down-regulates pro-cytokines synthesis and is associated with amelioration of the inflammatory response[14-16] and fibrosis[17-18]. The present study also found the trend from the pathological sections that IL-10 could relieve the degree of inflammation. In this experiment, we detected TGF-β1 in hepatic fibrosis group as well as in IL-10 treatment group.

TGF-β1 is a multi-functional cytokine in the liver involved in regulation of liver growth and induction of hepatocyte apoptosis, and is the most important medium involved in fibrotic and cirrhotic liver[19-22]. It can promote the development of liver fibrosis by inducing the synthesis of ECM proteins and down-regulating the expression of matrix, thus degrading enzymes and stimulating synthesis of their respective inhibitors[23-26]. The present study showed that, with the development of hepatic fibrosis, TGF-β1 increased in hepatic fibrosis rats and decreased after treatment with IL-10. TGF-β1 mRNA level in HSCs did not run parallel with the progression of hepatic fibrosis in the first stage. TGF-β1 protein level detected by immunohistochemistry and ELISA in IL-10 treatment group was lower than that in normal control group in the second stage. These results suggest that TGF-β1 is closely correlated with hepatic fibrosis and that the improvement of hepatic fibrosis is related with the decreasing expression of TGF-β1. These results also suggest that the expression of TGF-β1 of HSCs cannot be regarded as a predictable factor for the degree of hepatic fibrosis and that the ability of HSCs to produce TGF-β1 is declined when fibrosis develops. While the results of ELISA proved that IL-10 exerted its inhibitory effects on the expression of TGF-β1 not only in liver but also in serum, suggesting that IL-10 can inhibit the expression of TGF-β1. However, further studies are needed to confirm whether the expression of TGF-β1 in normal liver inhibited by IL-10 has any adverse effects and whether the effect of IL-10 on TGF-β1 is modulated by weakening the signal transduction.

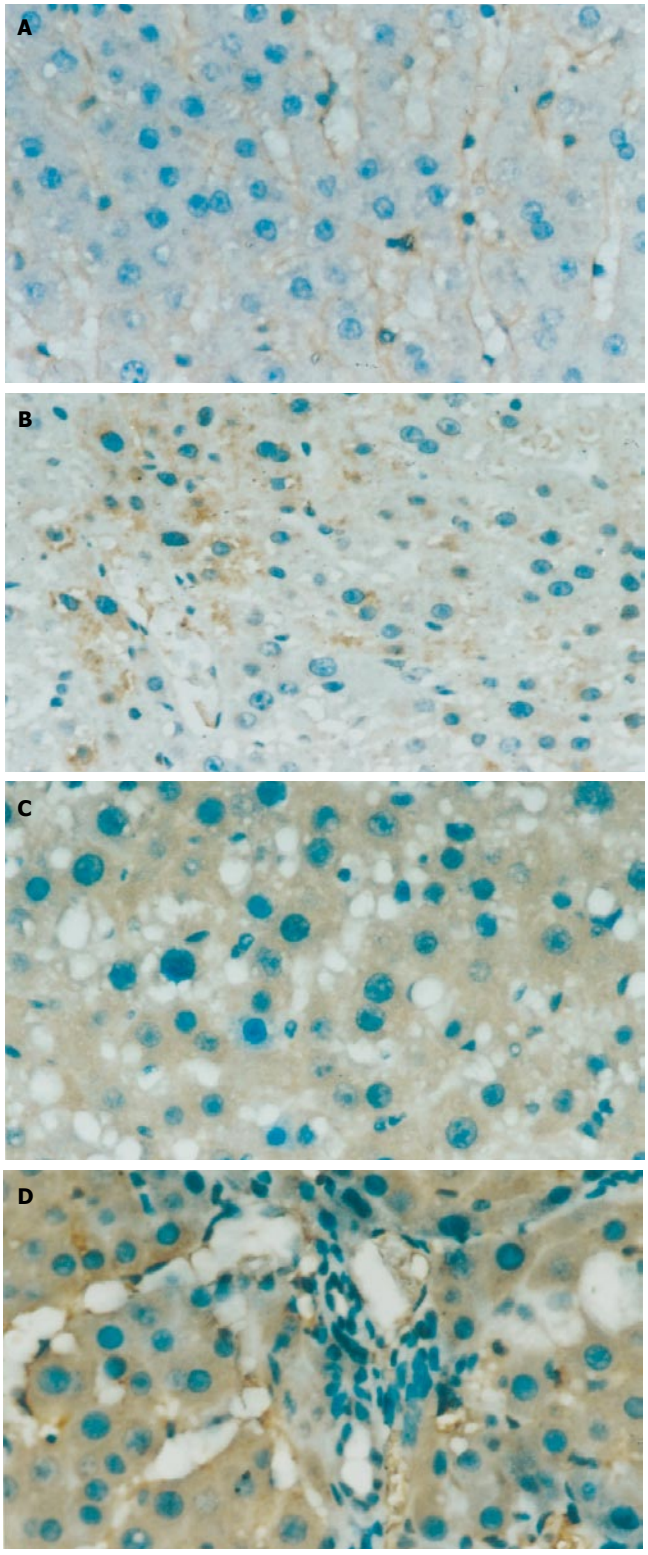

Figure 5 Expression of TGF-β1 in liver tissue in group N_2 (**A**), group T (**B**), group R (**C**), and group M (**D**).

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BASIC RESEARCH

Biological role of surface Toxoplasma gondii antigen in development of vaccine

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Abstract

AIM: To analyze the biological role of the surface antigen of *Toxoplasma gondii (T gondii)* in development of vaccine.

METHODS: The surface antigen of *T gondii* (SAG1) was expressed *in vitro*. The immune response of the host to the antigen was investigated by detection of specific antibody reaction to SAG1 and production of cytokines. Mice were immunized with recombinant SAG1 and challenged with lethal strain of *T gondii* RH. The monoclonal antibody to r-SAG1 was prepared and used to study the effects of SAG1 on *T gondii* tachyzoites under electromicroscope.

RESULTS: The mice immunized with recombinant SAG1 delayed death for 60 h compared to the control group. The recombinant SAG1 induced specific high titer of IgG and IgM antibodies as well as IFN-γ, IL-2 and IL-4 cytokines in mice. In contrast, IL-12, IL-6 and TNF-α were undetectable. When *T gondii* tachyzoites were treated with the monoclonal antibody to r-SAG1, the parasites were gathered together, destroyed, deformed, swollen, and holes and gaps formed on the surface.

CONCLUSION: SAG1 may be an excellent vaccine candidate against *T gondii*. The immune protection induced by SAG1 against *T gondii* may be regulated by both hormone- and cell-mediated immune response.

Key words: *Toxoplasma gondii;* Recombinant SAG1; Monoclonal antibody; Cytokines; Morphology change

Liu KY, Zhang DB, Wei QK, Li J, Li GP, Yu JZ. Biological role of surface Toxoplasma gondii antigen in development of vaccine. *World J Gastroenterol* 2006; 12(15): 2363-2368

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INTRODUCTION

Toxoplasma gondii (T gondii) is an intracellular coccidian parasite and causes the most common parasitic disease of animals and human beings[1]. The definitive hosts for the parasite are members of the Felidae family. The clinical manifestations associated with feline toxoplasmosis are anorexia, weight loss, lethargy, dyspnoea, ocular signs, pyrexia, vomiting and diarrhea, jaundice, myositis and abortion. Humans become infected when they ingest the Toxoplasma at infective stages (oocysts and tissue cysts) found in some cat feces and in raw meats. People weak in immune function may develop severe diseases such as encephalitis, pneumonia or other life-threatening conditions. Infants born with congenital toxoplasmosis may develop permanent diseases such as mental retardation or eye, liver and brain diseases. In cirrhotic patients, Toxoplasma IgG and IgM antibody positivity is as high as 68.5%[2]. In patients with AIDS, *T gondii* colitis can occur[3]. In veterinary medicine, *T gondii* infection may influence economics due to neonatal loss in sheep and goats[4], or as a source of transmission to humans[5]. Thus, it is of great value to develop an effective vaccine against *T gondii*.

The characterization of the molecules which play the role in the pathogenesis and immune protection is the important step in vaccine development. The surface of *T gondii* is the first component to contact with the host cells and the surface antigen of the parasite is recognized as the major study target. It was reported that there are 5 proteins in the superfamily of the surface antigens (SAG) of *T gondii*, including SAG1, SAG2 (22 Ku), p23, p35, and SAG3 (43 Ku). SAG1 is a 30 Ku glycoprotein and is therefore designated as SAG1/P30[6] and can be detected in the tachyzoite and sporozoite stages[7,8]. It was reported that SAG1 can elicit a lethal inflammatory process in mouse model of pathogen-driven ileitis[9]. However, the biological role of this surface protein remains unclear.

In the current study, the biological function of SAG1 was studied through the analysis of the induction of specific antibody, elicitation of specific cytokines by the recombinant protein for SAG1 (r-SAG1 or r-P30) of *T gondii*. The immune protection ability of SAG1 was studied by challenging experiments. The results show that SAG1 is a very important protein and its biological function is regulated by multiple mechanisms.

MATERIALS AND METHODS

Parasites and antigens

T gondii RH tachyzoites were maintained by two weekly passages of tachyzoites to peritoneum of BALB/C mouse. Four days later parasites in the peritoneal fluid were collected and the cavity was washed with 5 mL of phosphate buffered saline (PBS). The total tachyzoite crude antigen was obtained from washed and pelleted tachyzoites, resuspended in PBS and freeze-thawed three times, then subjected to 2 cycles of ultrasound disruption (UTR200) for 10 min and incubated at 37 ℃ for 2 h with 1% decanoyl-N-methylglucamide (MEGA 10, Sigma). After centrifugation at 36 000 r/min for 30 min, the pellet was discarded and the supernatant was aliquoted and stored at -70℃. The protein concentration was determined by BCA assay (Pierce) using BSA as standard.

Cloning and expression of SAG1 gene in E. coli

About 5×10^7 *T gondii* RH strain tachyzoites were concentrated by centrifugation, washed with PBS, then lysed in 0.1 mol/L Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulphate (SDS), 0.1 mol/L NaCl and 10 mmol/L EDTA and then treated with proteinase K (100 μg/mL) at 55℃ for 2 h. The genomic DNA was extracted by phenol/chloroform method followed by ethanol precipitation. After centrifugation the pellet was dissolved in TE buffer (10 mmol/L Tris-HCl, pH 8.0 and 1 mmol/L EDTA) and used as a template for polymerase chain reaction (PCR) amplification, which used the primers (5' TGGtttcactcttaagtgccctaaaacagc-3'and 5' ctgcattaacctgc agccccggcaaactc-3') together with PCR buffer, dNTP and Taq polymerase. The amplified SAG1 gene was inserted into the *Nco*I and *Hind*III sites of the plasmid pET-30a, and expressed as a His-tag fusion protein in *E. coli* BL21 (DE3) strain according to the manufacturer's instructions. The transformed bacteria were centrifuged and lysed by a combination of detergent Triton X-100, lysozyme and ultrasonication. The suspension was centrifuged and the pellet was dissolved in 8 mol/L urea solution containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L dithiothreitol (DTT) and 1 mmol/L EDTA. One hour after incubation at room temperature (RT), the supernatant was contrifuged and dialyzed at 4℃ followed by 2 mol/L urea solution at 4℃ for 1 h each. Dialysis was done twice in 50 mmol/L Tris-HCl (pH 8.0) with 1 mmol/L DTT at 4℃ and each lasting for 1 h. This was followed by overnight dialysis at 4℃ in the same buffer. The dialyzed sample was centrifuged and the supernatant was recovered and used as antigen.

Immunization and challenge

Five to 7-wk-old female BALB/c mice (purchased from Shandong University) housed under approved conditions of the animal research facility, were used in this study. Twenty-one BALB/c mice were immunized at two locations at the base of the tail with 30 μg of *T gondii* recombinant SAG1 in 0.1 mL of saline, which was emulsified with an equal volume of complete Freund's adjuvant (CFA) (Sigma, USA). Sixteen mice were injected with PBS. Booster was done 2 wk after the first injection using Freund's incomplete adjuvant for the r-SAG1 group. Sera were collected at 3 wk post immunization for antibody test. For cytokine test, mice were killed at 6 wk post immunization. The mice inoculated with r-SAG1 of *T gondii* were challenged intraperitoneally with 1×10^5 tachyzoite forms of *T gondii* RH strain 9 wk after the first immunization.

ELISA

To measure antigen-specific antibodies, plates were coated overnight at 4℃ with 10 μg/mL solution of antigen in 0.05 mol/L potassium phosphate buffer pH 8 (50 μL per well). Blocking was carried out with 2% BSA in PBS (pH 7.2) for 2 h at RT. After washed with PBS containing 0.05% Tween 20 (PBST20), sera were diluted in 1% BSA-PBST20 (50 μL per well) and incubated for 1 h at RT. After washed, bound antibodies were detected by incubation at RT for 1 h with horseradish peroxidase-labeled goat anti-mouse immunoglobulins at 1:2 000 dilution in 1% BSA-PBST20 (50 μL per well). Peroxidase activity was revealed by adding 50 μL per well of a solution containing 12.5% H_2O_2, 0.1 mol/L citrate-phosphate pH 4 and 10 mg/mL of TMB. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4 and the absorbance (A) was read at 450 nm in an ELISA microplate reader (Bio-Rad, USA). A sample was considered positive when A > A mean + 3 SD (cut off), where A is that of the tested sample, and A mean and SD are the mean and the standard deviation of the A of the sample from PBS, respectively.

SDS-PAGE and immunoblotting

SDS-PAGE was performed on 12% polyacrylamide gels according to the procedure of Laemmli[10] using a total tachyzoite extract or r-SAG1. Gels were transferred to nitrocellulose membranes as previously described[11] and blocked in PBS containing 5% nonfat dry milk. Membranes were incubated with primary Abs and then with anti-species alkaline phosphatase conjugates, both were diluted in PBS containing 1% nonfat dry milk. The alkaline phosphatase activity was detected with the ProtoBlot nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate color development system (Promega, Madison, WI).

Cytokine determination

At 6 wk post immunization, mice were sacrificed and single-cell suspension was prepared as previously described[12]. A volume of 10 μg/mL of r-SAG1 of *T gondii* was added and the supernatants were collected from cultures after 72 h and assayed by ELISA for cytokines.

The monoclonal antibody (mAb) was used for assay of IL-6 (MP5-20F3), IL-2 (JES6-1A12), IL-12 (9A5), IL-4 (BVD4-1D11), TNF-α (TN3-19.12), IFN-γ (AN-18) (BD Pharmingen).

Preparation of monoclonal antibodies

Monoclonal antibodies against recombinant SAG1 of *T gondii* were obtained by fusion of SP2/0 myeloma cells with the spleen cells of BALB/c mice immunized with r-SAG1 in the presence of polyethylene-glycol (Sigma) according to standard protocols. Hybrid cells were cultured in hypoxantine, aminopterin and thymidine (HAT) in DMEM/20% FBS medium. Clones growing in selected media were expanded and tested for antibody production. Positive clones were cloned twice by limiting dilution. Hybridomas were screened for specific antibody production by ELISA and immunoblot against the *T gondii* antigen. The identification of mAb isotype was made by ELISA using goat anti-mouse IgG subclasses (Nordic) appropriately diluted in PBS containing 0.1% Tween-20, 2% normal goat serum, followed by incubation with alkaline phosphatase conjugated rabbit anti-goat IgG (Sigma). Hybrid cells producing mAbs were injected to mouse intraperitoneally and after 2 wk ascites fluid was collected.

Electron microscopy

T gondii strains were harvested from BALB/C mice and washed twice with 0.05 mol/L pH7.2 PBS. The ascites fluid mAb against r-SAG1 was added (1:100) to the parasites. After incubation at 37℃ for 1h, the parasites were washed 3 times with PBS and fixed in 4% paraformaldehyde, 0.05% glutaraldehyde in cacodylate buffer+3% sucrose for 2 h at RT. After dehydration and embedding, the slides were observed under electron microscope (Hitachi 600, Hitachi, Japan).

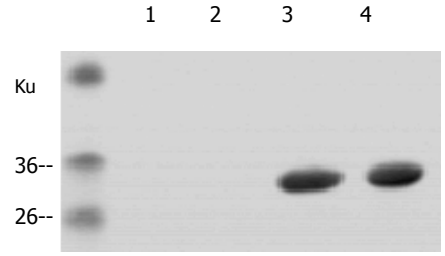

Figure 1 A strong band at 30 ku position after reaction with serum from mice infected with T. r-SAG1 of *Toxoplasma gondii* tachyzoites. The band was electrophoresed on SDS-12% polyacrylamide gel and then transferred onto a nitrocellulose membrane probed with sera (diluted at 1:100) from PBS-inoculated mice (lanes 1 and 2) or from mice infected with crude antigens of *T. gondii* (lanes 3 and 4). Bound antibodies were detected using anti-mouse-alkaline phosphatase conjugate. The positions of molecular masses are shown on the left.

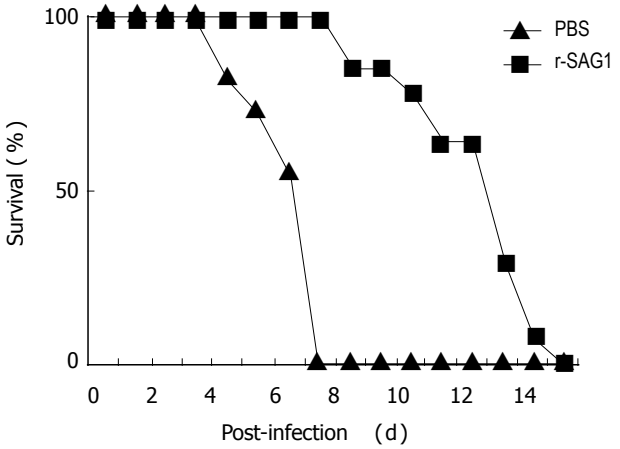

Figure 2 Protection of r-SAG1 against infection with tachyzoites of *Toxoplasma gondii*. Sixteen BALB/C mice were immunized with r-SAG1 of *T. gondii* and 11 mice were injected with PBS as a control. The mice were challenged with $1X10^5$ tachyzoites of *Toxoplasma gondii* RH at 9 wk post immunization. The number of the dead mice was counted.

RESULTS

Characterization of SAG1 recombinant protein

To study the biological function of the *P30* gene encoding SAG1, the *P30* gene of *T gondii* RH strain was amplified by PCR, the PCR product was subcloned into bacterial expression vector pET-30a and SAG1 was expressed as Histidin-fusion protein in *E. coli*. The gene was sequenced and the recombinant SAG1 was analyzed by Western blotting. A strong band at 30 Ku position was found after reaction with serum from mice infected with *T gondii* (Figure 1), indicating that the *P30* gene could encode a functional protein.

Immunization with r-SAG1 protein could protect mice from a lethal T gondii challenge

To test whether r-SAG1 could afford protection against a lethal *T gondii* RH strain infection, BALB/c mice were immunized with r-SAG1 as described in the methods. Nine weeks after the first injection, the animals were intraperitoneally infected with 1×10^5 tachyzoites of the highly virulent RH strain. The death number of the mice was counted after challenge. It was found that after challenge with RH tachyzoites, mice started to die on day 4 in PBS-inoculated control group. On day 7, all the mice in control group were dead. However, mice immunized with r-SAG1 delayed death for 60 h compared to those in the control group (Figure 2).

r-SAG1 induced specific antibody response

To investigate whether r-SAG1 could induce humoral immune response, the sera from mice were collected 3 wk after the last immunization. The titer and classes of immunoglobulin were tested by ELISA. It was obvious that vaccination of the mice with r-SAG1 induced a strong antibody response. The highest reaction antibody was immunoglobulin G (IgG). IgM was detected with a lower titer than that of IgG. IgA antibody response to r-SAG1 was very weak (Figure 3).

To test the specificity of the reaction of the antigen and antibody, the crude antigen of *T gondii* tachyzoites was separated on 12% SDS-PAGE and reacted with one of the mouse sera immunized with r-SAG1 by Western blotting.

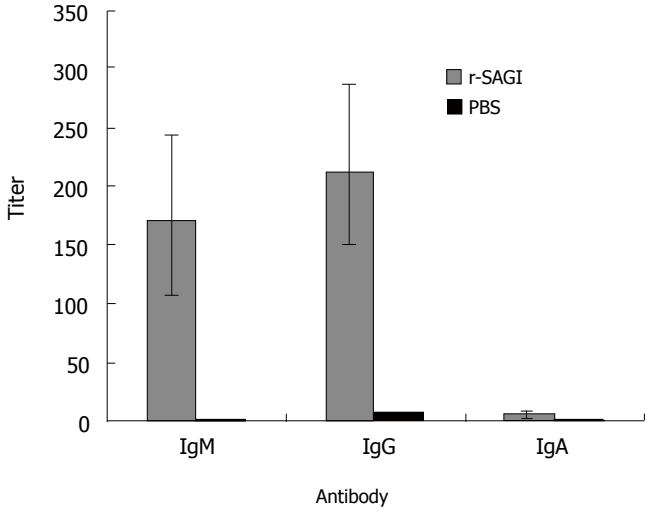

Figure 3 Antibody responses to r-SAG1. BALB/C mice were immunized with 30 μg r-SAG1 of *T gondii* or with PBS as a control. Booster was done with Freund's incomplete adjuvant 2 wk after the first injection. Three weeks post-immunization, sera were collected from 5 mice of each group. Antibodies were tested individually by ELISA.

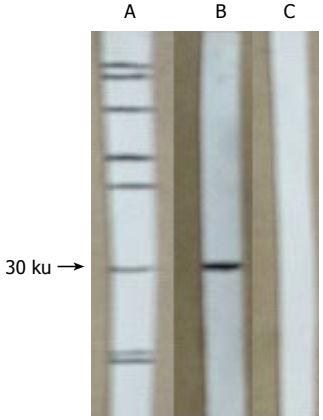

Figure 4 Profile of cytokines induced by r-SAG1 in PBS-inoculated mice (**A**) or r-SAG1 of *T gondii* immunized mice (**B**). Crude antigens of Toxoplasma gondii tachyzoites were electrophoresed on SDS-12% polyacrylamide gel and then transferred onto a nitrocellulose membrane probed with sera (diluted at 1:100). Bound antibodies were detected using anti-mouse-alkaline phosphatase conjugate. The positions of molecular masses are shown on the left.

A strong band was present on the 30 Ku position (Figure 3), corresponding to the predicted size from amino acid sequence.

Profile of cytokines induced by r-SAG1

To investigate the possibility of cell immune response induced by r-SAG1, the spleens of the mice immunized by r-SAG1 were isolated and the cytokines in the spleen cell suspensions were detected by ELISA. Compared to the control group, the spleen cells from r-SAG1-vaccinated BALB/c mice produced significant high levels of IFN-γ, IL-2 and IL-4, while IL-12, IL-6 and TNF-α were undetectable in splenocyte supernatants from all experimental and control animals analyzed (Figure 4).

Morphology change of T. gonddi tachyzoites caused by r-SAG1 antibody

To further elucidate the biological function of r-SAG1, monoclonal antibodies against r-SAG1 were prepared. Two specific clones against r-SAG1 were obtained and

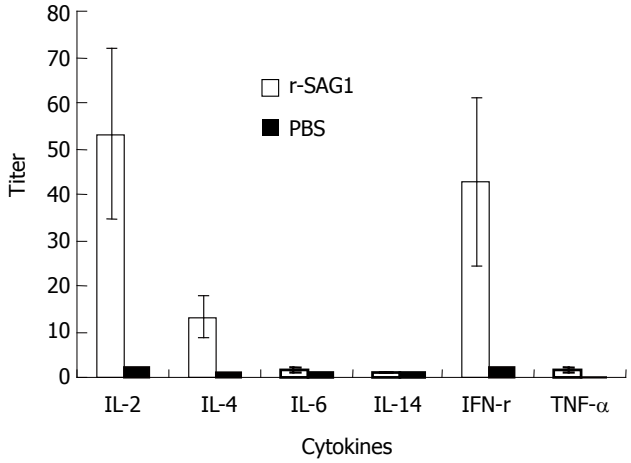

Figure 5 Production of cytokines induced by r-SAG1. BALB/C mice were immunized with r-SAG1 of *T gondii* or with PBS as a control. Six weeks after immunization, cytokines were tested individually by ELISA in 5 mice of each group.

designated as mAb2B3 and mAb1H6, respectively. Both clones were IgM isotype. The antibody produced a specific band at 30 Ku in Western blotting with *T gondii* crude antigens and r-SAG1 antigen.

To study the morphology effects of r-SAG1 antibody on *T gondii*, *T gonddi* tachyzoites were treated with specific antibody mAb2B3 and the morphology was observed under electron microscope. Compared to the PBS-treated control group, *T gondii* tachyzoites were gathered together and some tachyzoites were destroyed, some parasites were deformed(Figure 5). The holes and gaps on the surface of tachyzoites as well as the swollen parasites were observed (Figure 6).

DISCUSSION

In the current study, the recombinant protein for SAG1 of *T gondii* was prepared and the biological function of SAG1 was studied through the investigation of the lethal protection of mice. The possible mechanism was studied by the observation of the induction of specific antibody, elicitation of specific cytokines and the reaction of SAG1 specific antibody with *T gondii* tachyzoites. The study showed that SAG1 elicited a strong immune protective reaction which might be controlled by multiple mechanisms.

This study showed that the mice immunized with r-SAG1 delayed death for 60 h when challenged with *T gondii* RH tachyzoites, demonstrating that SAG1 is an important antigen of *T gondii*. The protection of the host against organism infection may involve many factors including specific antibody. It was reported that antibodies can regulate toxoplasma tachyzoites and thereby promote their killing by normal macrophages[13]. Kang *et al*[14] have proved that antibodies can block infection of host cells. In addition, antibodies in the presence of complement can kill extracellular toxoplasmas[15]. In the present study, the morphology changes of the parasite, such as agglutination, deformation, swelling hole and gap formation on the

Figure 6 Effects of monoclonal antibody to r-SAG1 on tachyzoites of *Toxoplasma gondii*. **A:** Agglutination of the parasites, hole and gap formation on the surface, destroyed parasites and membrane thickness; **B:** Deformed parasite; **C:** Swollen parasites. *Toxoplasma gondii* tachyzoites were collected from mice and washed with PBS. Monoclonal antibody mAb2B3 to r-SAG1 from mouse ascites fluid was diluted and reacted with the parasites and observed under electro-microscope at 6800 x.

surface, corresponding to SAG1 antibody treatment indicated that antibody might play an important role in the protection. The inference was verified further by the specific antibody production following the immunization of r-SAG1. After three weeks of vaccination with r-SAG1, high titer specific IgG and IgM were detected and the high titer of the antibodies was detected (data not shown). IgM antibodies could persist for at least 50 d and IgG could persist for an even longer time[16]. Therefore, when the mice were challenged at 9 wk, both IgG and IgM could contact with parasites to destroy the parasites, inhibit the infection of host cells, and thereby protect the mice from death.

In this study, the expression of cytokines such as IFN-γ, IL-4 and IL-2 was up-regulated in SAG1 immunized mice compared with control group. It was reported that IFN-γ induces inflammatory responses, thus limiting parasite proliferation[17]. In patients with alveolar echinococcosis, IFN-γ is higher, which might play a role in immunological defense against the parasite infection[18]. IL-2 (-/-) mice are unable to generate a protective IFN-γ response following infection with *T gondii* while IL-2 (-/-) mice have an intrinsic defect in their ability to activate and expand IFN-γ, producing T cells required for resistance to *T gondii*[19]. The production of IL-4 and IFN-γ allows identification of the Th0, Th1, or Th2 orientation of helper T cell clones. The production of antigen-specific cytokines suggests that SAG1 is able to elicit anti-SAG1 specific CD4+T-cells. Cell immune response is involved in the protection of the parasites. In addition, IFN-γ producs CD8+CTL which plays a prominent role in controlling *T gondii* infection[20]. It is possible that at least part of the detected IFN-γ can lead to CD8+CTL activation. However, further experiments are required to elucidate this question.

In conclusion, SAG1 induces dominant antibody response and a strong Th1-like T cell response characterized by high titer IFN-γ production during infection. The protective role of SAG1 may be controlled cooperatively by humoral and cellular immune response. The evidence of SAG1 protection against challenge of the parasite proves that SAG1 is a good vaccine candidate for the control of toxoplasmosis.

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BASIC RESEARCH

Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To investigate the effects of filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) on liver fibrosis induced by carbon tetrachloride (CCl_4) in rats.

METHODS: Forty Wistar rats were divided randomly into control group and model group. All model rats were given 200 mL/L CCl_4 (2 mL/Kg, po) twice a week for 8 wk. Four weeks after CCl_4 treatment, thirty model rats were further divided randomly into 3 subgroups: CCl_4 and two FMAC subgroups. Rats in CCl_4 and 2 FMAC subgroups were treated with FMAC 0, 0.5 and 1.0 g/kg, daily via gastrogavage beginning at the fifth week and the end of the eighth week. Spleen weight, blood synthetic markers (albumin and prothrombin time) and hepatic malondialdehyde (MDA) and hydroxyproline (HP) concentrations were determined. Expression of collagen I, tissue inhibitor of metalloproteinases (TIMP)-1 and transforming growth factor β1 (TGF-β1) mRNA were detected by RT-PCR. Histochemical staining of Masson's trichrome was performed.

RESULTS: CCl_4 caused liver fibrosis, featuring increased prothrombin time, hepatic MDA and HP contents, and spleen weight and decreased plasma albumin level. Compared with CCl_4 subgroup, FMAC subgroup (1 g/kg) significantly decreased the prothrombin time (36.7 ± 7.2 and 25.1 ± 10.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$) and increased plasma albumin concentration (22.7 ± 1.0 and 30.7 ± 2.5 in CCl_4 and FMAC groups, respectively, $P < 0.05$). Spleen weight was significantly lower in rats treated with CCl_4 and FMAC (1 g/kg) compared to CCl_4 treated rats only (2.7 ± 0.1 and 2.4 ± 0.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$). The amounts of hepatic MDA and HP in CCl_4 ± FAMC (1 g/kg) subgroup were also lower than those in CCl_4 subgroup (MDA: 3.9 ± 0.1 and 2.4 ± 0.6 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; HP: 1730.7 ± 258.0 and 1311.5 ± 238.8 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$). Histologic examinations showed that CCl_4 + FMAC subgroups had thinner or less fibrotic septa than CCl_4 group. RT-PCR analysis indicated that FMAC (1 g/kg) reduced mRNA levels of collagen I, TIMP-1 and TGF-β1 (collagen I: 5.63 ± 2.08 and 1.78 ± 0.48 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TIMP-1: 1.70 ± 0.82 and 0.34 ± 0.02 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TGF-β1:38.03 ± 11.9 and 4.26 ± 2.17 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$) in the CCl_4-treated liver.

CONCLUSION: It demonstrates that FMAC can retard the progression of liver fibrosis induced by CCl_4 in rats.

Key words: *Antrodia camphorata*; Liver fibrosis; Carbon tetrachloride

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INTRODUCTION

Antrodia camphorata is a new species of the genus *Antrodia* (*Polyporaceae*) parasitic in the inner cavity of the endemic species *Cinnamomum kanehirai* Hay[1]. Traditionally, it has been used as a remedy for food-, alcohol-, drug-intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer among Chinese. The growth rate of natural *A. camphorata* in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged culture method to obtain useful cellular materials, or to produce effective substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies. In Taiwan, several biotechnology companies have developed the submerged culture method for *A. camphorata*. In the market of Taiwan, the yield of mycelia or culture filtrate of fermented mycelia

is dependent on the different biotechnology companies. Preliminary pharmacological studies revealed that the antioxidant abilities of the culture filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) were correlated with their total polyphenols content based on the evaluation of different antioxidant system[2].

Liver fibrosis is the common end stage of most chronic liver disease regardless of the etiology[3], and its progression leads to cirrhosis and liver cancer. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of the free radical and lipid peroxides has attracted considerable attention[4]. It has been found that the metabolism of CCl_4 involves the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum[5]. CCl_4 is capable of causing liver lipid peroxidation, resulting in liver fibrosis[6]. Hsiao *et al*[7] reported that *A. camphorata* extract exerted protection against chronic chemical-induced hepatic injury in mice. In addition, Song *et al*[8] showed that FMAC possessed a protective activity against acute liver injury induced by CCl_4. However, the effect of FMAC in chronic liver disease is still unknown. In the present study, we attempted to assess the effect of FMAC on chronic CCl_4-induced liver fibrosis in rats.

MATERIALS AND METHODS

Preparation of test substance

FMAC was provided by Food Industry Research & Development Institute, Hsinchu, Taiwan. Culture of *Antrodia camphorata* BCRC 930032 was inoculated onto potato dextrose agar (PDA) and incubated at 30 ℃ for 15 to 20 d. The whole colony was then cut and put into the bottle with 50 mL sterile water. After homogenization, the fragmentated mycelia suspension was used as inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 r/min with aeration rate of 0.2 vvm and temperature of 30 ℃. A 5-d culture of 15 L mycelia inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation condition was the same as the seed fermentation but operating with an aeration rate of 0.075 vvm. The deep red culture filtrate was separated from the broth harvested at the 331st hour and poured through the non-woven fabric on a 20-mesh sieve. FMAC was concentrated about 20 fold (450 g/L) under reduced pressure at 50 ℃, and stored at -30 ℃ until use. FMAC was suspended in distilled water and administered orally to each rat at a volume of 10 mL/kg body weight.

Since antioxidant and anti-radical properties of plant extracts have been attributed to most phenolic compounds, it is expected that the effectiveness of the extracts is related to their phenolic content[9]. To guarantee the reproducibility of pharmacological experiments, the phenolic compounds in FMAC were determined by a modification of the method of Barness *et al*[10] using catechin as the standard. The concentration of phenolic groups in FMAC was 39.71 μg/mg.

Animals

Male Wistar rats were obtained from the National Laboratory of Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory chow and tap water *ad libitum*. The experimental animals were housed in air-conditioned room of 21-24 ℃ with 12 h of light. The rats were allowed free access to powdered feed, and main water that was supplied through an automatic watering system. When they reached 250-300 g, the rats were used for experiments. Rats were divided randomly into control and model groups according to the body weight in proper range one day before administration of the test substance. All animals received humane care and the study protocols were in compliance with our institution's guidelines for use of laboratory animals.

CCl_4-induced liver fibrosis

Fibrosis was induced in thirty rats by an oral administration of 2 mL/kg body weight of 200 mL/L CCl_4 (diluted in olive oil) twice a week for 8 wk. At the end of 4 th wk after CCl_4 treatment, the CCl_4-treated rats were further divided into 3 subgroups based on the plasma alanine aminotransferase (ALT) level, since the plasma ALT is the major parameter for liver injury. The plasma ALT levels for normal control and 3 CCl_4-treated subgroups were 675 ± 62, 8856 ± 1321, 9005 ± 1659 and 8208 ± 1324 (nkat/L), respectively. The animals received CCl_4 with distilled water or FMAC (0.5, 1.0 g/kg; *po*, daily) which was added at the last four wk of the treatment. The time interval between CCl_4 and FMAC administrations were 5 h to avoid the disturbance of absorption of each other. After blood was drawn from rats at the eighth week, the animals were sacrificed at the same time and the liver and spleen were quickly taken off. They were then weighed after being clearly washed with cold normal saline and sucked up of the moisture. The largest lobe of liver was divided into four parts, and the same parts were 1) submerged in 40 g/L neutral formaldehyde for the preparation of pathological section; 2) after weighed, the liver was completely dried at 100 ℃ for the determination of collagen content; 3) the samples for RT-PCR analysis were kept in liquid nitrogen; 4) other sample was stored at -80 ℃ until assay.

Assessment of liver functions

The blood was centrifuged at 4700 r/min (Jouan BR4i, France) at 4 ℃ for 15 min to separate the plasma. The levels of plasma ALT and albumin were assayed using clinical test kits (Roche Diagnostics) spectrophotometrically (Cobas Mira; Roche, Rotkreuz, Switzerland). Prothrombin time was measured using a coagulation analyzer (Sysmex-CA1000) and reagent (Dade thromboplastin C plus).

Assays of hepatic lipid peroxidation and hydroxyproline

Livers were homogenized in nine volumes of ice-cold 0.15 mol/L KCl, 1.9 mmol/L ethylenediaminetetraacetic acid. The homogenate was used for the determination of lipid peroxidation. Lipid peroxidation was measured by the methods of Ohkawa *et al*[11] using 2-thiobarbituric acid. The lipid peroxidation was expressed as malondialdehyde (MDA) μmol/g protein. Protein was measured by the method of Lowry *et al*[12] using bovine serum albumin as the standard. Hydroxyproline (HP) determination fol-

Table 1 Primer sequences for PCR amplification

mRNA		Primer sequence	Length (bp)
Collagen I	Sense	5' CGA CTA AGT TGG AGG GAA CGG TC 3'	182
	Antisense	5' TGG CAT GTT GCT AGG CAC GAC 3'	
TIMP-1	Sense	5' TCC CTT GCA AAC TGG AGA GT 3'	140
	Antisense	5' GTC ATC GAG ACC CCA AGG TA 3'	
TGF-b1	Sense	5' TAT AGC AAC AAT TCC TGC CG 3'	162
	Antisense	5' TGC TGT CAC AGG AGC AGTG 3'	
GAPDH	Sense	5' CTT CAT TGA CCT CAA CTA CAT GGT CTA 3'	99
	Antisense	5' GATG ACA AGC TTC CCA TTC TCA G 3'	

Table 2 Effect of FMAC on plasma albumin concentration and prothrombin time in CCl_4-treated rats

Group	Dose (g/kg per d)	Albumin (g/L)	Prothrombin time (sec)
Control	–	36.0 ± 1.3	17.7 ± 0.9
CCl_4 + H_2O	–	22.7 ± 1.0[b]	36.7 ± 7.2[b]
CCl_4 + FMAC	0.5	23.1 ± 5.1	28.5 ± 9.9
	1	30 .7± 2.5[a]	25.1 ± 10.2[a]

[a]$P < 0.05$ *vs* CCl_4 + H_2O group; [b]$P < 0.01$ *vs* control group.

Table 3 Effect of FMAC on spleen weight, hepatic malondialdehyde and hydroxyproline contents in CCl_4-treated rats

Group	Dose (g/kg per d)	Spleen (g)	Malondialdehyde (μmol/g protein)	Hydroxyproline (μg/g tissue)
Control	–	1.1 ± 0.1	1.9 ± 0.1	645.0 ± 64.5
CCl_4 + H_2O	–	2.7 ± 0.1[d]	3.9 ± 0.1[d]	1730.7 ± 258.0[d]
CCl_4 + FMAC	0.5	2.8 ± 0.2	2.7 ± 0.1	1741.5 ± 257.1
	1	2.4 ± 0.2[a]	2.4 ± 0.6[b]	1311.5 ± 238.8[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* CCl_4 + H_2O group; [d]$P < 0.001$ *vs* control group.

lowed a method designed by Neuman *et al*[13]. Dried liver tissue after hydrolysis was oxidized by H_2O_2 and colored by p-dimethylaminobenzoaldehyde and absorbance was determined at 540 nm. The amount of HP is expressed in mg/g wet tissue.

RNA extraction and RT-PCR analysis

Total RNA was isolated from livers of the rats using the acid guanidum thiocyanate-phenol-chloroform extraction methods as described by Chomczynski *et al*[14]. Five micrograms of total RNA from each liver sample were subjected to reverse transcription (RT) by MMuLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the reverse transcription mix were used for amplification by polymerase chain reaction (PCR) of fragments specific to collagen I, transforming growth factor (TGF)-β1 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 using the primer pairs listed in Table 1. The levels of expression of all the transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. PCR product was run on a 20 g/L agarose gel recorded by polarid film; bands were quantitated by densitometer.

Pathological examinations

For histopathological examination, the formalin-fixed liver was embedded in paraffin, cut into 4-5 μm thick sections, stained with Masson's trichrome. Fibrosis was graded according to the method of Ruwart *et al*[15], grade 0: normal liver; grade (1) increase of collagen without formation of septa; grade (2) formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); grade (3) complete but thin septa interconnecting with each other, so as to divide the parenchyma into separate fragments; grade (4) as grade 3, except with thick septa (complete cirrhosis). To avoid sampling error, all biopsies were obtained from the same lobe and these semi-quantitative grades were performed without knowledge of sample treatment.

Statistical analysis

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were treated by one-way analysis of variance using the Dunnett's test. Liver histopathological examination data were analyzed by the Kruskall-Wallis non-parametric test, followed by a Mann–Whitney *U*-test. The significance level was set at $P < 0.05$.

RESULTS

Concentrations of plasma albumin and prothrombin time

The plasma albumin concentrations were lower in rats given CCl_4 than that in the control group (Table 2). While in the rats treated by FMAC (1 g/kg), the levels of plasma albumin was markedly higher than that in the CCl_4 model group. The prothrombin time in the CCl_4 model group was much longer than that in control group. FMAC (1 g/kg) significantly shortened the prothrombin time.

Weights of spleen

Marked splenomegaly was caused by CCl_4 treatment; the weight of spleen in the CCl_4-treated group was about 245% of the control group (Table 3). The increase of spleen weight by CCl_4 treatment was significantly reduced by FMAC (1 g/kg).

Liver MDA and HP contents

CCl_4 induced liver fibrosis to the rats resulting in a marked increase of hepatic MDA and HP contents (Table 3). FMAC (1 g/kg) treatment significantly reduced the increase of hepatic MDA and HP contents caused by CCl_4.

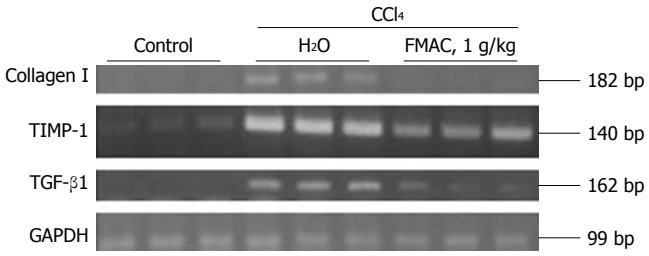

Figure 1 Effect of FMAC on the hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats.

Table 4 Effect of FMAC on hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats

Group	Dose (g/kg per d)	Collagen I: GAPDH ratio	TGF-β1: GAPDH ratio	TIMP-1: GAPDH ratio
Control	–	0.68 ± 0.55	0.38 ± 0.18	0.14 ± 0.08
CCl_4 + H_2O	–	5.63 ± 2.08[b]	38.03 ± 11.9[b]	1.70 ± 0.82[b]
CCl_4 + FMAC	0.5	3.94 ± 1.18	28.90 ± 7.22	0.58 ± 0.02
	1	1.78 ± 0.48[d]	4.26 ± 2.17[d]	0.34 ± 0.02[d]

[b]$P < 0.001$ *vs* Control group; [d]$P < 0.01$ *vs* CCl_4 + H_2O group.

RT-PCR analysis of liver tissue

Fragments specific to collagen I, TIMP-1 and TGF-β1 were amplified by RT-PCR (Figure 1). The values from densitometric analysis, after normalization against the corresponding GAPDH transcript were expressed as the collagen I/GAPDH, TIMP-1/GAPDH and TGF-β1/GAPDH ratios. The levels of collagen I, TIMP-1 and TGF-β1 mRNA in rat liver were significantly increased by CCl_4 treatment (Table 4), while the administration of FMAC (1 g/kg) significantly decreased the levels of collagen I, TIMP-1 and TGF-β1 mRNA.

Pathological examination

CCl_4 induced liver damage of the rats. Masson's stain showed clear nodular fibrosis at the central vein and the portal vein area (Figure 2B). Treatment of FMAC (1 g/kg) showed marked improvement of these pathological changes of the tissues (Figure 2C and Table 5).

DISCUSSION

The results of the present study indicate that even after the initiation of hepatic fibrosis in a rat model of CCl_4-induced liver damage, FMAC administration reduced liver fibrosis, as demonstrated by smaller increases in hepatic collagen and lower mRNA expression of collagen I compared with CCl_4 model group. These effects were mainly observed when FMAC was administered from wk 5 to wk 8 of CCl_4 treatment. Both plasma albumin and blood clotting factors were mainly synthesized in the liver. When the chronic liver damage led to fibrosis, the albumin contents dropped and prothrombin time prolonged[16,17]. In this experiment, CCl_4 induced chronic liver lesions in rats and there appeared a decrease of plasma albumin and an increase of prothrombin time. FMAC clearly counteracted both the decrease of albumin content in the plasma and the prolongation of prothrombin time. These results showed that FMAC ameliorated the decline of liver synthetic functions caused by chronic liver injuries.

Liver fibrosis or cirrhosis leads to blockage of blood flow into the liver and causes portal hypertension and it also influences the blood flow of spleen and gives rise to splenomegalia[18]. CCl_4 in this experiment induced chronic

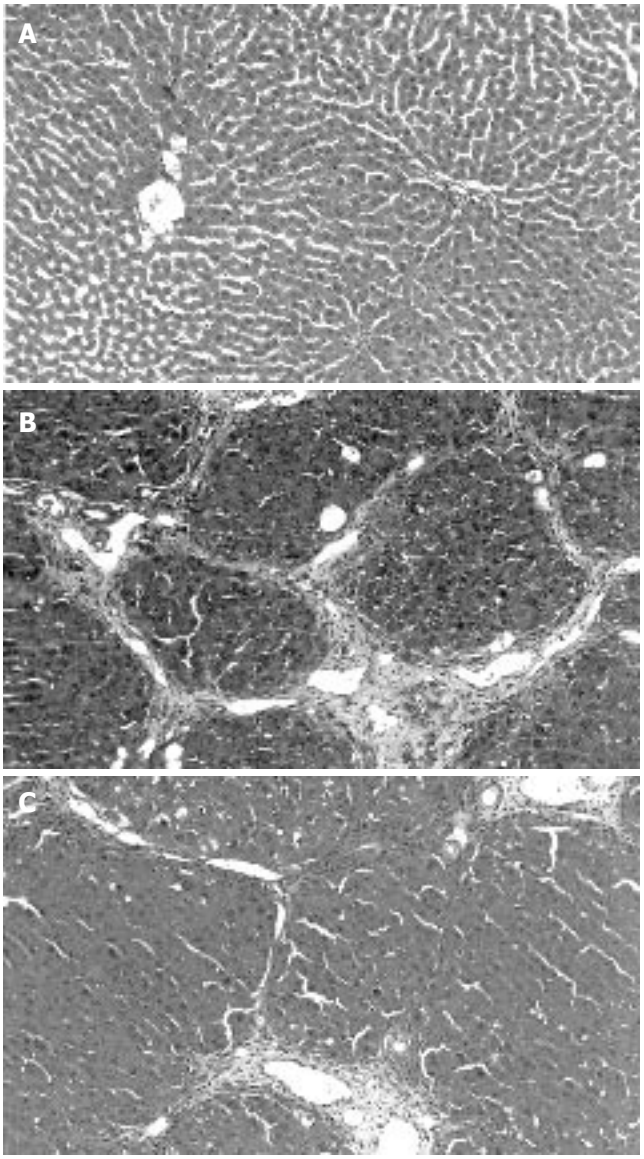

Figure 2 Liver histopathology of rats (Masson's stain). **A**: control group; **B**: CCl_4 + H_2O group, showing micronodular formation and complete septa interconnection with each other; **C**: CCl_4 + FMAC (1 g/kg) group, showing a marked reduction in fiber deposition. Scale bar = 50 μm.

Table 5 Effect of FMAC on CCl4-induced liver fibrosis in rats

Group	Dose (g/kg per d)	Score of hepatic fibrosis 0	1	2	3	4	Average
Control	–	10	0	0	0	0	0
CCl_4 + H_2O	–	0	0	3	6	1	2.8 ± 0.6
CCl_4 + FMAC	0.5	0	1	3	5	1	2.7 ± 0.8
	1.0	0	4	5	1	0	1.7 ± 0.7[b]

[b]$P < 0.01$ *vs* CCl_4 + H_2O group.

hepatic fibrosis as well as splenomegalia. FMAC could improve splenomegalia, indicating that it might ameliorate portal hypertension.

It is well known that liver fibrosis is a result of increased collagen synthesis[19], and HP is the unique component in collagen[19]. The amount of collagen can be reflected by the contents of HP and can be used to express the extent of fibrosis[19]. When CCl_4 was applied in this experiment to induce liver fibrosis, the content of HP in liver obviously increased. FMAC could reduce the content of HP, which was confirmed by the histopathological examinations. Many studies have shown that level of collagen I increases during liver fibrosis[20]. Therefore, we also investigated the effect of FMAC on the mRNA expression of collagen I. Treatment with FMAC was effective in reducing the amount of collagen I mRNA expression. This result further confirmed that FMAC could remit hepatic fibrosis.

Regardless of the etiologic factors, gross remodeling of extracellular matrix in the fibrotic liver is regulated by a balance of synthesis and enzymatic degradation of extracellular matrix[21]. Matrix degradation is catalyzed by the activity of matrix metalloproteinases. The activities of matrix metalloproteinases are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The expression of TIMPs drastically increased or decreased with time during liver fibrogenesis and fibrosis resolution, respectively[22]. Four members of the TIMP family have been characterized so far and designated as TIMP-1 to TIMP-4[23]. It has been suggested that TIMP-1 plays an important role in the pathogenesis of liver fibrosis[24]. Consistent with previously published work[25], we observed elevated levels of TIMP-1 upon treatment with CCl_4. Treatment with FMAC was effective in reducing the level of TIMP-1 expression, indicating liver fibrosis resolution might be enhanced. This result supported that FMAC could suppress liver fibrotic progression caused by CCl_4.

TGF-β1 is a profibrogenic cytokine, because it directly stimulates extracellular matrix production by both Kupffer cells and stellate cells[26,27]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[28,29]. Blockade of TGF-β1 synthesis or signaling is a primary target for the development of antifibrotic approaches and modern hepatology has facilitated the design of drugs removing this causative agent[30]. In this experiment, CCl_4 treatment increased, while FMAC significantly reduced TGF-β1 mRNA expression. This result suggested that FMAC ameliorated liver fibrosis perhaps by reducing TGF-β1 secretion.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl_4 hepatotoxicity[5]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl_4 administration[6]. Our results confirmed these findings that hepatic lipid peroxidation is increased during hepatic fibrogenesis. We also found that FMAC inhibited CCl_4-induced hepatic lipid peroxidation. These results indicated that FMAC might inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis. A large number of studies indicated that FMAC is a good free radical scavenger[3, 31].

In conclusion, the present study has demonstrated that FMAC retards the progression of liver fibrosis in CCl_4-treated rats possibly by scavenging free radicals formed in the liver. It may be expected that FMAC has preventive potentials in liver fibrosis.

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BASIC RESEARCH

Expression and activity of inducible nitric oxide synthase and endothelial nitric oxide synthase correlate with ethanol-induced liver injury

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Abstract

AIM: To study the expression and activity of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in rats with ethanol-induced liver injury and their relation with liver damage, activation of nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) expression in the liver.

METHODS: Female Sprague-Dawley rats were given fish oil (0.5 mL) along with ethanol or isocaloric dextrose daily via gastrogavage for 4 or 6 wk. Liver injury was assessed using serum alanine aminotransferase (ALT) activity and pathological analysis. Liver malondialdehyde (MDA), nitric oxide contents, iNOS and eNOS activity were determined. NF-κB p65, iNOS, eNOS and TNF-α protein or mRNA expression in the liver were detected by immunohistochemistry or reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS: Chronic ethanol gavage for 4 wk caused steatosis, inflammation and necrosis in the liver, and elevated serum ALT activity. Prolonged ethanol administration (6 wk) enhanced the liver damage. These responses were accompanied with increased lipid peroxidation, NO contents, iNOS activity and reduced eNOS activity. NF-κB p65, iNOS and TNF-α protein or mRNA expression were markedly induced after chronic ethanol gavage, whereas eNOS mRNA expression remained unchanged. The enhanced iNOS activity and expression were positively correlated with the liver damage, especially the necro-inflammation, activation of NF-κB, and TNF-α mRNA expression.

CONCLUSION: iNOS expression and activity are induced in the liver after chronic ethanol exposure in rats, which are correlated with the liver damage, especially the necro-inflammation, activation of NF-κB and TNF-α expression. eNOS activity is reduced, but its mRNA expression is not affected.

Key words: Alcoholic liver disease; Inducible nitric oxide synthase; Endothelial nitric oxide synthase; Nuclear factor-κB

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INTRODUCTION

Nitric oxide (NO) has been recognized as an important mediator of physiological and pathophysiological processes. It is produced by at least two isoforms of nitric oxide synthase (NOS) in the liver, such as eNOS and iNOS. eNOS is a Ca^{2+}- and calmodulin-dependent constitutive isoform and plays an important role in vasorelaxation, whereas iNOS is not a constitutive enzyme and its expression may be induced by stimuli such as lipopolysaccharide or proinflammatory cytokines[1,2].

The role of NO in alcohol-induced liver injury still remains controversial. Nanji *et al*[3] reported that arginine, a substrate for NO, can significantly attenuate ethanol-induced liver injury. Treatment with N-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, enhances alcohol-induced liver injury in the Tsukamoto-French enteral rat model[4]. However, iNOS knockout mice or wild-type mice treated with N-(3-aminomethyl) benzyl-acetamindine (1400W), a highly selective iNOS inhibitor, are protected against liver damage caused by alcohol[5]. Uzun *et al*[6] showed that L-NAME might produce a restorative effect on ethanol-induced liver damage.

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that plays an important role in regulation of inflammatory responses. NF-κB is composed of homo- and hetero-dimers of five members of the Rel family, including NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B, and Rel C. The most prevalent activated form of NF-

κB is a heterodimer consisting of a p50 or a p52 subunit and p65. NF-κB exists in cytoplasm in an inactive form associated with regulatory proteins called IκB. After stimulation, it is translocated to the nuclei and bound to decameric DNA sequences, and activates transcription of target genes[7]. NF-κB has been shown to be functionally important for iNOS induction[8]. In the present study, we used fish oil plus ethanol gavage model of alcoholic liver disease, and examined the expression and activity of iNOS and eNOS in the liver, and their relation with liver damage, activation of NF-κB and TNF-α expression.

MATERIALS AND METHODS

Chemicals and reagents

Polyclonal rabbit anti-iNOS and anti-NF-κB p65 were obtained from Santa Cruz Biotechnology, Inc. Biotinylated goat-anti-rabbit IgG was purchased from Beijing Zhongshan Reagent Corp. TRIzol reagent was purchased from Invitrogen. DL2000 DNA ladder marker was from TaKaRa Biotech Co., Ltd. M-MLV reverse transcriptase and its buffer, deoxyribonucleotide (dNTP, 10 mmol/L) and oligo(dT)$_{15}$ primer were from Promega Corp. Taq DNA polymerase and its buffer, rRNasin ribonuclease inhibitor was from Biostar. Polymerase chain reaction (PCR) primers for eNOS, iNOS, TNF-α and GAPDH, were synthesized by Sai-Bai-Sheng Biocompany (Shanghai, China). Malondialdehyde (MDA), nitric oxide (NO) and nitric oxide synthase (NOS) activity assay kits were purchased from Nanjing Jiancheng Bioengineering Co.Ltd, China.

Animal model

Female Sprague-Dawley rats weighing 200-250 g, were obtained from the Experimental Animal Center of Wuhan University. After acclimation for 6-7 d, animals were randomly divided into 4-wk dextrose group (n = 5), 6-wk dextrose group (n = 5), 4-wk ethanol group (n = 8), and 6-wk ethanol group (n = 8). Rats were given 0.5 mL fish oil along with ethanol or isocaloric dextrose intragastrically by gavage. The initial dose of ethanol was 6 g/kg per day (solutions maximally containing 56 mL/100 mL alcohol), and the dose was progressively increased during wk 1 to a maintenance dose of 8 g/kg per day that was continued for another 3 or 5 wk. All rats had free access to regular standard rat chow throughout the experiment. The animals were weighed three times per wk. At the end of the experiment, the animals were anaesthetized with urethane (20%, 1.0 g/kg) and sacrificed by bleeding from femoral arteries and veins. Blood samples were collected. Immediately after exsanguination, the livers were harvested. Small portions of the liver were kept at -70 ℃ for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, whereas another portion was separated and immersed in 10% buffered formalin solution for histological and immunohistochemical examination. All animals were given humane care in compliance with the institutional guidelines.

Pathological evaluation

Liver specimens, 1.0 cm×0.5 cm×0.3 cm in size, were processed for light microscopy. This processing consisted of fixing the specimens in 10% formaldehyde for 12-24 h, embedding them in paraffin, slicing sections of 5 μm in thickness and staining the sections with hematoxylin and eosin. Histological assessment was performed by a pathologist unaware of the study. The severity of liver pathology was assessed as follows[9]: steatosis (the percentage of liver cells containing fat), 1+, <25% of cells containing fat; 2+, 26%-50% of cells containing fat; 3+, 51%-75% of cells containing fat; and 4+, >75% of cells containing fat. Necrosis was evaluated as the number of necrotic foci/mm^2 and inflammation was scored as the number of inflammatory cells/mm^2.

Serum alanine aminotransferase assay

Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000 r/min for 10 min and kept at -20 ℃ before determination. Enzymatic activity of alanine aminotransferase (ALT) was measured using a commercial kit by an RA 1000 automatic biochemical analyzer (Japan).

Liver MDA contents and NOS activity assay

Liver samples were thawed, weighed and homogenized 1 : 9 w:v in 0.9% saline. Then the homogenates were centrifuged at 3 000 r/min for 10 min at 4 ℃ and the supernatant was taken for the assays of MDA contents, NOS activity and total protein.

MDA was assayed by measuring the thiobarbituric acid-reactive substances (TBARS) levels spectrophotometrically at 532 nm. Results were expressed as nmol.mg^{-1} protein.

NOS catalyzed the formation of NO and L-citrulline from L-arginine and molecular oxygen, and NO reacted with a nucleophile to generate color compounds. The absorbance at 530 nm NOS activity was calculated and expressed as U/mg protein. One unit of NOS activity was defined as the production of 1 nmol nitric oxide per second per mg tissue protein. Total NOS activity was measured as follows: 10% tissue homogenate (100 μL) was incubated with 200 μL substrate buffer, 10 μL reaction accelerator and 100 μL color development reagent at 37 ℃ for 15 min after mixing. Then 100 μL clearing reagent and 2 mL stop solution were added, mixed and absorbances were read at 530 nm. For measuring iNOS activity, an inhibitor was added before incubation according to the manufacturer's instructions.

Total protein concentration was determined using the Coomassie blue method with bovine serum albumin as standard.

Liver NO assay

Liver samples were thawed, weighed and homogenized 1 : 9 w:v in 0.9% saline. The homogenates were then centrifuged at 1 000 r/min for 5 min at 4 ℃, the supernatant was taken for NO assay and total protein determination.

NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO_3^- plus NO_2^-) and the stable end products of NO metabolism. In the procedure nitrate was enzymatically converted into nitrite by the enzyme nitrate reductase, followed by quantitation of nitrite using

Table 1 PCR primers used for iNOS, eNOS, TNF-α and GAPDH

Name	Sense	Antisense	Product length (bp)
iNOS	TTCTTTGCTTCTGTGCTAATGCG	GTTGTTGCTGAACTTCCAATCGT	1061
eNOS	TGGGCAGCATCACCTACGATA	GGAACCACTCCTTTTGATCGAGTTAT	202
TNF-α	GCCAATGGCATGGATCTCAAAG	CAGAGCAATGACTCCAAAGT	357
GAPDH	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	309

Griess reagent at the absorbance of 550 nm as previously described[10]. Results were expressed as μmol/g protein.

Immunohistochemical detection of iNOS and NF-kB p65 in liver

Five μm thick sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 3% H_2O_2 in deionised water for 5-10 min. Nonspecific binding sites were blocked by incubating the sections in 10% normal rabbit serum for 10-15 min. The sections were then incubated with polyclonal rabbit anti-iNOS (dilution 1:25) or anti-NF-kB p65 (dilution 1:100) overnight at 4 ℃, followed by incubation with biotinylated goat-anti-rabbit IgG at room temperature for 10-15 min. After 3×3 min PBS rinses, the horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10-15 min. The antibody binding sites were visualized by incubation with a diaminobenzidine-H_2O_2 solution. The sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in cytoplasm or nuclei were recognized as positive staining for iNOS or NF-kB p65 respectively. NF-kB immunoreactivity was expressed as the number of positive cells/high-power field (× 400).

RT-PCR analysis of iNOS, eNOS and TNF-α mRNA expression in liver

Total RNA was isolated from approximatively 50-100 mg snap-frozen liver tissue using the TRIzol protocol as suggested by the supplier. Following precipitation, the RNA was resuspended in RNAse-free buffer, the concentration was assayed by measuring ultra-violet light absorbance at 260 nm and purity was estimated from the ratio of A_{260}/A_{280}.

Single-stranded complementary DNA (cDNA) was synthesized from the total RNA using the following method. In brief, 2 μg RNA was preincubated with 0.5 μg oligo(dT)$_{15}$ primer and diethylpyrocarbonate (DEPC)-treated water was added to a total volume of 15 μL at 70 ℃ for 5 min, then rapidly chilled on ice. To the annealed primer/template 5 μL M-MLV 5×reaction buffer, 1.25 μL dNTP (10 mmol/L, each), 25 units of rRNasin ribonuclease inhibitor, 200 units of M-MLV RT and DEPC-treated water were added to a final volume of 25 μL. The reaction was incubated at 42 ℃ for 60 min and terminated by placing it on ice after deactivation at 85 ℃ for 5 min. The resulting cDNA was used as a template for subsequent PCR.

The PCR mixture contained 5 μL of 10×Taq buffer, 1 μL of dNTP (10 mmol/L, each), 1 μL of gene specific primers (Table 1, sense and anti-sense primers, 25 pmol/μL, each), 2.0 units of Taq DNA polymerase and 1 μL of cDNA in a total volume of 50 μL. Thirty-five cycles of amplification were performed with initial incubation at 94 ℃ for 3 min and a final extension at 72 ℃ for 7 min, each cycle consisted of denaturation at 94 ℃ for 45 s, annealing at 54 ℃ for 45 s and extension at 72 ℃ for 1 min. To ensure the use of equal amounts of cDNA from each group samples in PCR, the aliquots of the reverse transcription products were used in PCR with the primers for house-keeping gene GAPDH. The quantities of cDNA producing equal amounts of GAPDH-PCR-product were used in PCR with the primers for iNOS, eNOS and TNF-α. Following RT-PCR, 5 μL samples of amplified products was resolved by electrophoresis on 2% agarose gel and stained with ethidium bromide. The level of each PCR product was semi-quantitatively evaluated using a digital camera and an image analysis system (Vilber Lourmat, France), and normalized to GAPDH.

Statistical analysis

Results were presented as mean ± SD unless otherwise indicated. Differences between groups were analyzed using analysis of variance with *post hoc* analysis using LSD test. The correlation was analyzed with Spearman's correlation coefficients. $P < 0.05$ was considered statistically significant.

RESULTS

In each of the four groups, the rats increased their weight at a constant rate. There was no difference in weight gain among the groups.

Pathological changes and serum aminotransferase activity

The animals given fish oil plus dextrose developed slight steatosis in the liver, but no obvious inflammation or necrosis was observed (Figure 1A). However, chronic ethanol gavage for 4 wk caused steatosis, minimal to mild inflammation and necrosis in the liver (Figure 1B). Prolonged ethanol administration (6 wk) enhanced the liver damage. Pronounced macrovesicular and microvesicular steatosis as well as spotty necrosis and mild inflammation were observed (Figure 1C, Table 2).

Consistent with the histological changes, serum ALT levels, an index of liver cell injury, were significantly increased in 4-wk ethanol group, and further increased in 6-wk ethanol group as compared with dextrose groups (Table 2).

Liver MDA and NO contents

Liver contents of MDA, a marker of lipid peroxidation, were significantly increased after 4 wk ethanol gavage

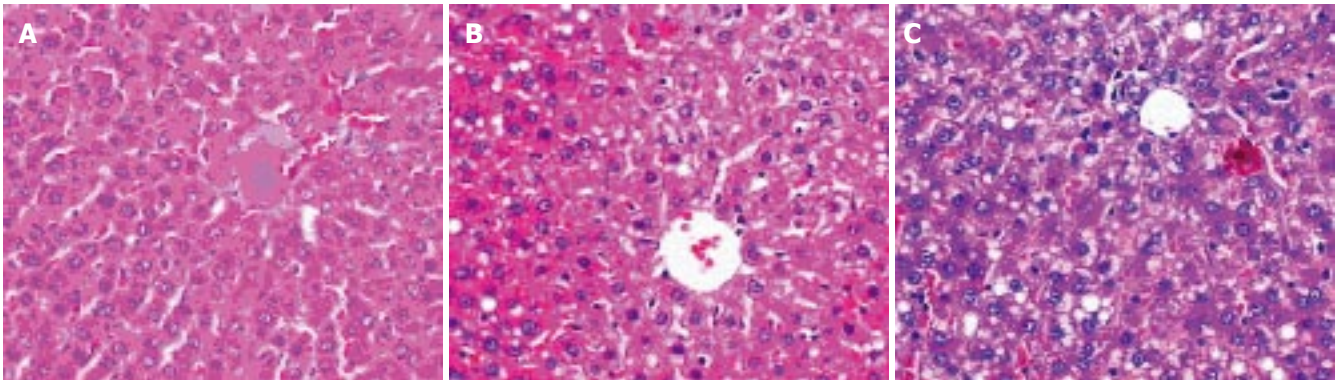

Figure 1 Representative pathologic changes in 4-wk dextrose group (**A**), 4-wk ethanol group (**B**), and 6-wk ethanol group (**C**). Original magnification, ×200.

Table 2 Pathological scores, serum ALT levels and liver MDA contents in different experimental groups (mean ± SD)

Groups	Fatty liver	Necroinflammation	ALT(U/L^{-1})	MDA(nmol/mg^{-1} protein)
4-wk dextrose	0.2±0.25	1.0±1.0	43.4±3.05	4.83±0.70
4-wk ethanol	2.1±0.64[a]	16.1±2.17[a]	117.3±6.92[a]	8.64±0.62[a]
6-wk dextrose	0.6±0.35	1.6±1.52	46.0±3.16	5.36±0.31
6-wk ethanol	2.9±0.64[b]	19.9±4.29[b]	124.1±7.28[b]	9.38±0.39[b]

[a]$P<0.05$ *vs* 4-wk dextrose group; [b]$P<0.05$ *vs* 6-wk dextrose group.

Table 3 Liver NO contents, iNOS and eNOS activity in different experimental groups (mean ± SD)

Groups	NO (μmol/g^{-1} protein)	iNOS (U/mg^{-1} protein)	eNOS(U/mg^{-1} protein)
4-wk dextrose	0.75±0.14	0.27±0.07	0.64±0.06
4-wk ethanol	1.67±0.15[a]	0.60±0.07[a]	0.48±0.03[a]
6-wk dextrose	0.87±0.07	0.35±0.06	0.58±0.04
6-wk ethanol	1.84±0.12[b]	0.70±0.09[b]	0.43±0.05[b]

[a]$P<0.05$ *vs* 4-wk dextrose group; [b]$P<0.05$ *vs* 6-wk dextrose group.

compared with dextrose groups. Prolonged ethanol exposure (6 wk) led to a further increase in MDA contents (Table 2).

Levels of NO in the liver of two dextrose groups were 0.75 ± 0.14 and 0.87 ± 0.07 μmol/g protein, respectively. Chronic ethanol gavage-induced NO level was two-fold higher in 4-wk ethanol group and further higher in 6-wk ethanol group (Table 3).

Liver NOS activity

The isoforms of NOS present in the liver were mainly eNOS and iNOS as previously reported[2]. The amount of total NOS activity minus iNOS activity might represent the activity of eNOS. Chronic fish oil plus ethanol gavage led to a marked elevation in iNOS activity with further elevation in 6-wk ethanol group compared with 4-wk ethanol group. In contrast, the eNOS activity was significantly reduced compared with dextrose groups (Table 3).

Expression of NF-κB p65 in liver

NF-κB p65 staining was present in cytoplasm and nuclei, but only nuclear staining was considered positive. There was no positive staining in two dextrose groups, whereas following chronic ethanol administration, remarkable enhancement in the positive staining was observed (Figures 2A and 2B). The number of positive cells in 4-wk and 6-wk ethanol groups was 8.0 ± 1.1 and 10.0 ± 1.9 /high-power field, respectively (Figure 2C). The NF-κB p65 positive cells were primarily Kupffer cells and hepatocytes.

iNOS, eNOS and TNF-α expression in liver

Only faint immunoreactive staining of iNOS was detected in the liver from dextrose groups (Figure 3A). However, intense staining of iNOS was observed in 4-wk ethanol group (Figure 3B), and more intense staining was found in 6-wk ethanol group (Figure 3C). The staining was mainly present in both severely damaged and perivascular areas.

iNOS mRNA was barely detectable in the liver of dextrose groups, but after chronic ethanol gavage (4 wk), iNOS mRNA was markedly induced. Prolonged ethanol gavage (6 wk) was associated with more intense bands (Figure 4). In dextrose groups only faint bands for TNF-α were detected. After chronic ethanol gavage, similar

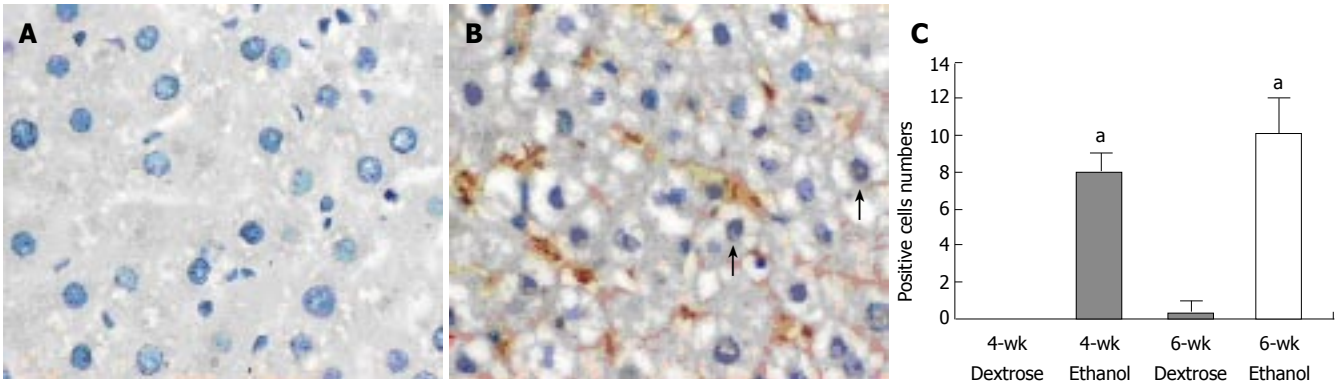

Figure 2 Expression of NF-κB p65 in 6-wk dextrose group (**A**), 6-wk ethanol group (**B**) and the number of positive cells in high-power fields (**C**). [a] $P < 0.01$ *vs* dextrose group. Original magnification, ×400.

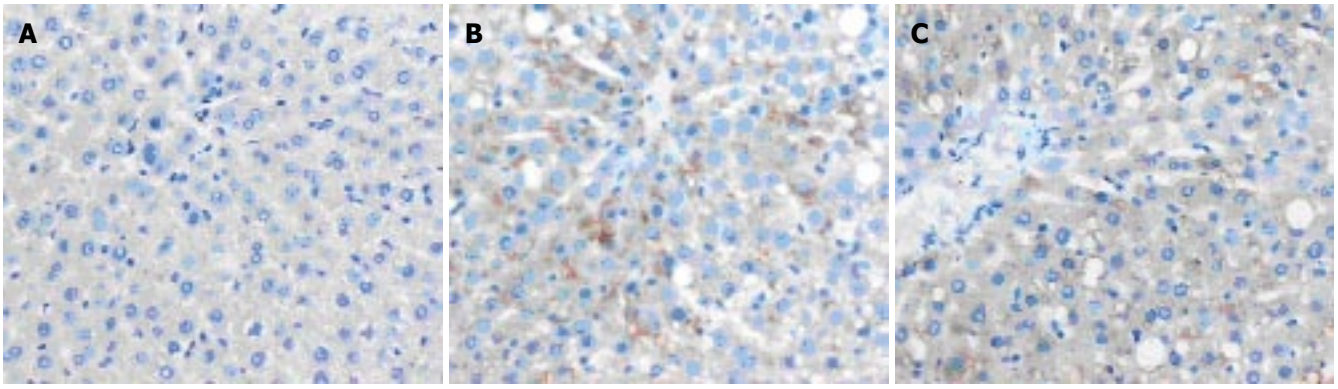

Figure 3 Immunohistochemical detection of iNOS in 6-wk dextrose group (**A**), 4-wk ethanol group (**B**), and 6-wk ethanol group (**C**). Original magnification, ×200.

expression pattern of iNOS was also observed in TNF-α mRNA expression (Figure 4).

In contrast, there was no significant change in eNOS mRNA expression between dextrose and ethanol groups (Figure 4).

Relationship between liver iNOS activity, expression and other parameters

Correlation analysis showed that liver iNOS activity was positively correlated with the severity of liver damage (steatosis, necroinflammation) ($r = 0.71$ and 0.93, respectively, $P < 0.05$), especially the necroinflammation. iNOS expression was only detected in rats with liver damage, activation of NF-κB and intense TNF-α mRNA expression. The intensity of the former paralleled that of the latter.

DISCUSSION

It has been reported that dietary fatty acids play an important role in the pathogenesis of alcoholic liver disease[11,12]. Polyunsaturated fatty acids enriched in fish oil promote alcoholic liver injury and pathological changes occur only in rats fed with ethanol containing polyunsaturated fatty acids[13]. Alcoholic liver injury is more severe and develops rapidly in women than in men[14]. Our study employed female rats and used fish oil plus ethanol gavage to make an animal model of alcoholic liver injury. The rats developed pathological changes in the liver after 4 or 6 wk, such as steatosis, spotty necrosis and inflammation, all of which resemble alterations found in clinical alcoholic liver disease. This chronic gavage of alcohol in rats is a simple experimental model that mimics key aspects of alcoholic liver disease in humans, and is useful for exploring the mechanism and treatment of alcoholic liver disease.

NO is an important biological mediator and has been shown to be involved in diverse physiological as well as pathological processes[1]. In our study, chronic ethanol gavage led to a significant elevation of liver NO contents compared to dextrose groups. NO is generated by NOS. We assayed the activity of total NOS and iNOS in liver. Because the main isoforms of NOS in the liver are eNOS and iNOS[2], the amount of total NOS activity minus iNOS activity may represent the activity of eNOS. Our study showed that iNOS activity was significantly elevated after chronic ethanol consumption in rats, whereas eNOS activity was markedly reduced as compared with dextrose groups. Accompanying the enhanced activity, iNOS expression detected by immunohistochemistry and RT-PCR in the liver was significantly increased in ethanol groups compared to dextrose groups. However, the eNOS mRNA expression was comparable between these groups. The results suggest that the elevated NO release in the liver is attributable to the enhanced activity and expression of iNOS. Relationship analysis showed that enhanced

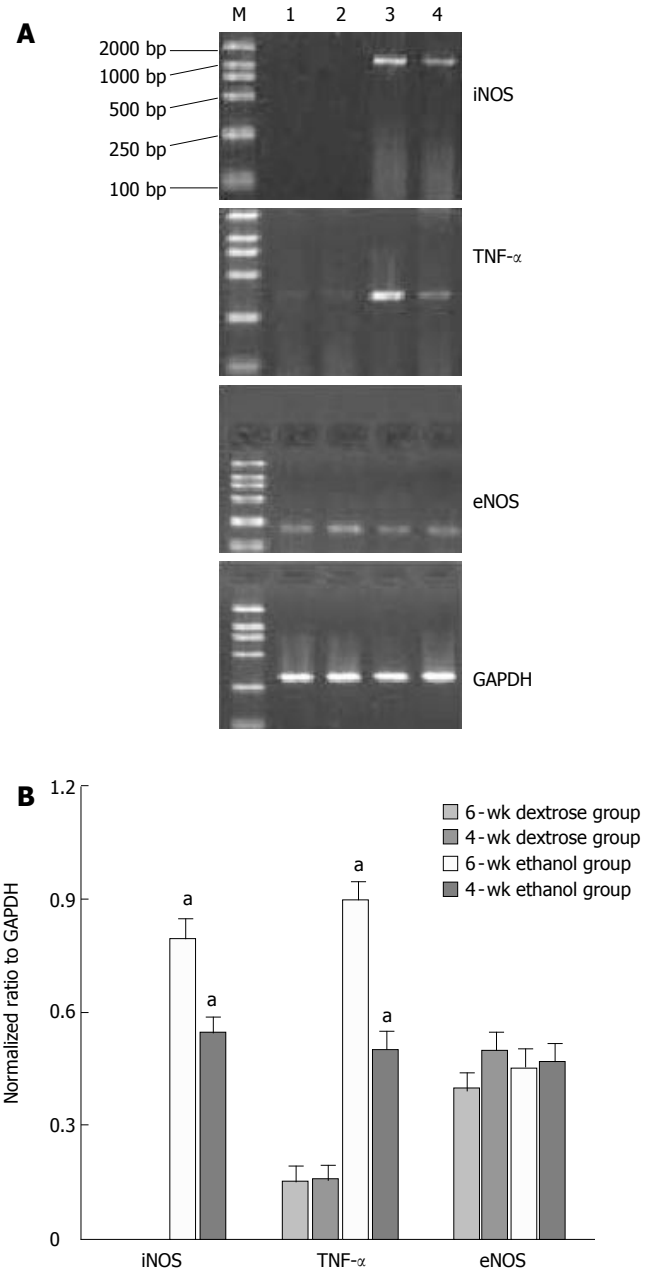

Figure 4 RT-PCR analysis of mRNAs for iNOS, eNOS and TNF-α in the liver. **A**: representative bands for iNOS, TNF-α, eNOS and GAPDH transcripts (lane 1: 6-wk dextrose group, lane 2: 4-wk dextrose group, lane 3: 6-wk ethanol group, lane 4: 4-wk ethanol group); **B**: normalized densitometric ratios of iNOS, TNF-α and eNOS transcripts to GAPDH. [a] $P<0.01$ *vs* dextrose group.

iNOS activity and expression were associated with the severity of liver damage, especially the necroinflammation, suggesting that iNOS contributes to alcohol-induced liver injury. It was reported that iNOS knockout mice or wild-type mice treated with 1400W, a highly selective iNOS inhibitor, are protected against alcohol-induced liver injury[5]. In contrast with iNOS, eNOS activity is reduced after treatment with ethanol. A recent report showed that chronic alcohol intake attenuates hepatic eNOS activity by increasing the expression of the inhibitory protein caveolin-1 and enhancing its binding to eNOS[15]. Treatment with L-NAME, the stronger eNOS inhibitor, exacerbates alcohol-induced liver injury[4]. These facts suggest that the role of NO in ethanol-induced liver injury may be dependent on the isoforms of NOS.

Oxidant stress has been reported to play a role in the pathogenesis of alcohol-induced liver injury[16]. In this study, liver contents of MDA, a marker of lipid peroxidation, were significantly elevated in the ethanol groups as compared with dextrose groups. Oxidant stress can result in degradation of the cytoplasmic NF-κB inhibitor, IκB, allowing translocation of NF-κB to nuclei[17]. Our study showed that chronic ethanol gavage significantly enhanced the expression of active NF-κB in the liver, as evidenced by the increased number of NF-κB p65 positively stained (in nuclei) cells. Furthermore, TNF-α mRNA expression in the liver was markedly increased. These results are consistent with the report of Nanji *et al*[18], who showed that NF-κB is activated during alcoholic liver disease in the presence of pro-inflammatory stimuli, resulting in increased expression of pro-inflammatory cytokines and chemokines. In our study, correlation analysis showed that iNOS expression was positively associated with NF-κB p65 expression, suggesting that increased iNOS expression may be caused by the activation of NF-κB. Activated NF-κB, a transcription factor, may bind to the specific DNA sequence of iNOS to promote its expression[19].

In conclusion, iNOS expression and activity induced in ethanol-induced liver injury are responsible for the elevated NO production. The induction of iNOS is associated with liver damage, especially necroinflammation, activation of NF-κB and elevated TNF-α mRNA expression in the liver.

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CLINICAL RESEARCH

Food intolerance and skin prick test in treated and untreated irritable bowel syndrome

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Abstract

AIM: To correlate the clinical features of treated and untreated patients with irritable bowel syndrome (IBS) to the results of skin prick test (SPT) for food and inhalant allergens.

METHODS: We recruited 105 subjects to form three different target groups: treated group ($n = 44$) undergoing treatment for IBS, untreated group ($n = 31$) meeting the Rome II criteria without treatment for IBS, control group ($n = 30$) with no IBS symptoms.

RESULTS: SPT results were different among the three groups in which SPT was positive in 17 (38.6%) treated patients, in 5 (16.1%) untreated patients and in 1 (3.3%) control ($P < 0.01$). The number of positive SPTs was greater in the IBS group than in the control group ($P < 0.001$). The number of positive food SPTs was higher in the treated IBS group than in the untreated IBS group ($P = 0.03$).

CONCLUSION: Positive food SPT is higher in IBS patients than in controls.

Key words: Irritable bowel syndrome; Skin prick test; Food allergy

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INTRODUCTION

Irritable bowel syndrome (IBS) is common. Depending on the studies, the prevalence (using Rome II criteria) varies between 9%-22% among Caucasians[1-3]. In Korea, the prevalence of IBS has been reported to be 6.6% and there is no difference in the prevalence between men and women[4].

In patients with IBS, postprandial worsening of symptoms as well as food-related reactions to one or more foods is very common[5]. In published data, speculation of an immunological reaction to foods in IBS has been reported[6,7]. Data from dietary elimination and food challenge studies support the role of diet in the pathogenesis of IBS[8-10]. This hypothesis is supported by the response to disodium cromoglycate in such patients[11,12]. Although well designed randomized controlled studies are scarce, milk, wheat, egg and foods high in salicylates or amines are consistently a problem among the studies[13,14]. Recently, Dainese *et al*[7] have indicated discrepancies between reported food intolerance and skin prick test (SPT) findings in IBS patients, suggesting that the SPT used to identify food sensitization mediated by the IgE mechanism is inappropriate. IBS is a heterogeneous disease having various symptoms and severity. So in our study, IBS patients were divided into treatment and untreated groups. Moreover, to clarify the possible role of food hypersensitivity in IBS, we used both common food and inhalant antigens. The results of SPT for food allergens were compared to the SPT for inhalant allergens.

The aim of this study was to correlate the clinical features of treated and untreated IBS patients to the results of the skin prick test for food and inhalant allergens, total IgE level, and radio- allegro-sorbent test (RAST)

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Institutional Review Board of the Hanyang University. Among the 480 medical students, 35 meeting the Rome II but not treated

Table 1 Characteristics of studied subjects, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)
Age (years)	37.9+13.2	27.2+1.9	25.8+1.7
Gender			
Male	19 (43.2)	16 (51.6)	22 (73.3)
Female	25 (56.8)	15 (48.4)	8 (26.7)
IBS subgroups			
C-IBS	10 (22.7)	12 (38.7)	-
D-IBS	15 (34.1)	9 (29.0)	-
A-IBS	19 (43.2)	10 (32.3)	-
Type of initial health care system		-	-
Tertiary center	13 (29.5)	-	-
Primary care	25 (56.8)	-	-
OB/Gyn [3]	1 (2.3)	-	-
Oriental-medicine	5 (11.4)	-	-
Reason for seeking for health care			
Abdominal pain	17 (38.7)	-	-
Discomfort, bloating	14 (31.8)	-	-
Altered stool pattern	13 (29.5)	-	-

C-IBS = Constipation-predominant IBS; D-IBS = Diarrhea-predominant IBS; A-IBS = Alternating constipation and diarrhea IBS.

for IBS and 35 randomly selected healthy undergraduate students were recruited in the study. After giving their informed consent, all volunteers underwent a physical examination and laboratory screening (upper gastrointestinal endoscopy, colonoscopy, routine red and white blood cell count, and biochemical examinations). Subjects receiving any medication (antihistamines, steroids, H_2 receptor antagonist, anti-inflammatory drugs, and herb medications) over a two-week period prior to study and those with current severe allergic disease identified by interview were excluded from the study (n = 9). Therefore, out of 70 students, 31 untreated IBS students and 30 healthy students participated in the trial. Forty-four consecutive patients with IBS referred to our institution for evaluation and treatment served as positive controls. Standard workup included upper gastrointestinal endoscopy, laboratory testing and colonoscopy.

The enrolled patients were divided into three groups after clinical evaluation and the application of Rome II criteria. Group I consisted of 44 patients with IBS referred to our institution for evaluation and treatment. All treated patients were selected from a single tertiary outpatient clinic (treatment group). Group II consisted of 31 untreated IBS patients meeting the Rome Ⅱ criteria but not treated for IBS (untreated group). Group III consisted of 30 healthy undergraduate students (control group).

Questionnaire and clinical measurements

To assess abdominal symptoms and intensity, all study participants were given a questionnaire. A modified WHO-QOL (WHOQOL-BREEF) method validated by Min *et al*[15] was used to measure the quality of life in the IBS and control groups. The questionnaire consisted of 26 items measuring four dimensions of health: physical health, psychological health, social relationships and environmental health. Each item asked the respondents to indicate the extent to which their IBS interfered with their health during the previous four weeks.

Skin prick test and RAST

SPT was carried out on all subjects on the upper back involving the application of 70 fresh food extracts using the prick-by-prick method. Some of the fresh food extracts used were saury, mackerel, beef, pork, chicken, milk, egg white, egg yolk, wheat flour, buckwheat, rice, beans, apples, peaches, tomatoes, celery, carrots, onions, peanuts, chocolate, and coffee. A doctor carried out an additional SPT involving the application of inhalant allergens. The 11 commercial allergens tested for D-pteronyssinus, D-farinae, alternaria alternata, grass pollen, tree pollen, mugwort pollen, willow pollen, ragwort, dog hair, cat fur, and cockroaches mix. In both SPTs, one drop of the extract was placed on the skin, and a disposable syringe needle was placed into the drop and then into the skin until a small puncture was visible. A histamine control was employed. SPT was considered positive if the net wheal diameter was significantly greater than the net wheal diameter of the histamine reaction. The reaction to the extract was measured 15-20 min after application. Discomfort and bleeding were not evident.

Serum samples for total IgE and total eosinophil count were collected by trained phlebotomists. Two 10 mL blood samples from an antecubital vein were taken. IgE measurement was determined by the paper radioimmunosorbent test (PRIST, Pharmacia Laboratories, USA) using a Phadebas IgE kit. The total IgE test provided evidence for IgE sensitivity to antigens because the IgE antibody resulted in mast cell activation and release of histamine. Also, total eosinophils were stained with a Hinkelman solution, and the eosinophil count provided immediate hypersensitivity to IgE antibody.

Statistical analysis

Tables were constructed for frequency and percentage. Categorical data were analyzed using the chi-square test or Fisher's exact test. Continuous data were analyzed using the Student's t-test and one-way ANOVA or Scheffe's test. To minimize type I errors, $P < 0.05$ was considered statistically significant. Exact P-values are listed in the tables and text. All statistical analyses were performed using the SPSS 11.0 statistical package.

RESULTS

Patient population

In the study population, 44 treated IBS patients had a mean age of 38 years (37.9±13.2), 31 untreated IBS patients had a mean age of 27 years (27.2±1.9), and 30 controls had a mean age of 26 years (25.8±1.7). There were no statistically significant differences in gender ratio, height, weight, alcohol and coffee intake, and smoking habits among the three groups (not shown). In the treated group, the number of females (56.8%, n = 25) was greater than that of males (43.2%, n = 19). In this group, the most subtype IBS was found in 19 patients. The subtype constipation dominant IBS was found in 12 patients of untreated group. Subjects in the treated group mainly sought for a primary care setting as the first visit due to

Table 2 Symptoms, duration of symptoms, and onset of IBS, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	*P*
Duration of symptom			0.035
Less than 1 h	5 (11.4)	7 (22.6)	
1-24 h	13 (29.5)	15 (48.4)	
More than 1 day	26 (59.1)	9 (29.0)	
Severity of subjective symptoms			0.001
Extremely severe	6 (13.6)	-	
Very	22 (50.0)	5 (15.6)	
Somewhat	14 (31.8)	19 (62.5)	
Not very	1 (2.3)	6 (18.8)	
Not at all severe	1 (2.3)	1 (3.1)	
Onset of IBS symptoms			0.065
Recent 6 mo	3 (6.8)	2 (6.3)	
1 year ago	8 (18.2)	1 (3.1)	
1-2 years ago	6 (13.6)	4 (12.5)	
2-5 years ago	13 (29.5)	12 (40.6)	
5-10 years ago	7 (15.9)	11 (34.4)	
10-20 years ago	5 (11.4)	1 (3.1)	
More than 20 years ago	2 (4.5)	-	

abdominal pain (15 patients), stool pattern change (13 patients) and abdominal bloating (13 patients) (Table 1).

Subgroup analysis and scores of quality of life (WHOQOL-BREEF)

We utilized a questionnaire to compare and estimate the onset, duration and severity of symptoms between the two IBS groups. More than half of the treated IBS patients (59.1%) reported that the symptoms lasted for more than 24 h, but only 29.0% of untreated IBS patients reported that the symptoms lasted for more than 24 h. Five IBS patients (11.4%) in the treated group complained of abdominal symptoms almost all day for more than one month. Subjective severity score of symptoms was also higher in the treated IBS patients than that of the untreated IBS patients ($P = 0.001$). But there was no significant difference in the onset of the disease between the two groups ($P = 0.065$) (Table 2).

Modified WHOQOL (WHOQOL-BREEF) questionnaire validated by Min *et al*[15] was used. It consisted of 26 items measuring four dimensions of health. All dimensions including overall quality of life scores were significantly lower in IBS patients than in controls (Table 3).

History of allergies and food intolerance

There was no significant difference in the reported food intolerance among the three groups. However, among the treated and untreated IBS patients, dairy products were reported to cause most of the intolerance. Of the treated and untreated IBS patients, 25 (56.8%) and 21 (67.7%) had an allergic condition and only 9 (30%) of the controls had an allergic condition ($P = 0.008$). However, no difference in the number of allergic conditions was found between the treated and untreated IBS patients. Rhinitis was the most common condition, and five patients had a history of asthma in the IBS patients (Table 4).

Skin prick test and IgE level

Seventy fresh food extract allergens were used to identify the cause of food hypersensitivity. SPT results were different among the three groups. SPT was positive in 17 (38.6%) treated IBS patients, in 5 (16.1%) untreated IBS patients, and in 1 (3.3%) control ($P < 0.01$). The number of positive SPTs was greater in the IBS patients than in the controls ($P < 0.001$). The number of positive food SPTs was greater in the treated patients than in the untreated patients ($P = 0.03$). The most frequent SPT positive food allergen was saury among the different groups. Five IBS patients had positive SPT for rice (Table 5). Eleven treated and untreated IBS patients and four controls had positive SPT for inhalant allergens. No significant difference in SPT inhalant allergens was noted among the three groups. The most common reactive inhalant allergens were D-farinae and D-pteronyssinus.

There were no significant differences in IgE and total eosinophil counts among the three groups. Also, no significant differences in IgE and total eosinophils were noted between the treated and untreated IBS patients (Table 6).

Table 3 Score of quality of life (mean ± SD)

Domain	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value[a]	*P* value[c]
Physical health	52.47±15.06	59.09±13.17	75.74±16.25	0.001	0.001
Psychological health	52.98±17.00	56.06±13.71	68.75±13.79	0.001	0.014
Social relationships	53.77±13.92	59.47±11.59	61.81±16.47	0.067	0.005
Environmental health	48.44±15.19	54.69±11.19	63.15±12.70	0.001	0.005
Overall QOL	5.79± 1.09	6.36± 1.33	8.21± 0.93	0.001	0.002

QOL: quality of life. [a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between IBS patients and control.

Characteristics according to the food SPT in treated and untreated groups

When SPT for food allergen and gender ratio were compared between those with a positive and negative SPT, no difference was noted. Also, there were no significant differences in positive SPT between IBS subtype and current allergy history. However, food intolerance and positive inhalant allergen were much higher in SPT positive food allergens ($P = 0.013$, $P = 0.006$). Twenty-two IBS patients had one or more skin positive tests. Serum samples were taken for RAST in order to compare the SPT results. Only three treated IBS patients had a positive RAST for beans, pork, and beef (Table 7).

DISCUSSION

The term "adverse food reaction" or "food-related symptoms" encompasses immunological responses and non-immunological responses to food. Food hypersensitivity/ allergy is used to describe conditions in which an immunological mechanism may be demonstrable[16,17]. In contrast, food intolerance is a non-immunological response to proteins that may result from particular constituents of foods such as toxins (e.g., food poisoning) or pharmacological agents (e.g., caffeine or tyramine) or from host factors such as lactase deficiency. However, food hypersensitivity

Table 4 History of allergies and food intolerance, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value [a]	*P* value [c]	*P* value [e]
History of allergies	25 (56.8)	21 (67.7)	9 (30.0)	0.017	NS	0.008
Atrophy	5 (11.4)	5 (16.1)	2 (22.2)			
Asthma	3 (6.8)	2 (6.5)	-			
Rhinitis	10 (22.7)	7 (22.6)	5 (55.6)			
Eczema	4 (9.1)	5 (16.1)	3 (33.3)			
Hives	4 (9.1)	7 (22.6)	-			
Reported food intolerance	36 (81.8)	27 (87.1)	23 (76.7)	NS	NS	NS
Dairy products	24 (54.5)	17 (54.8)	11 (36.7)			
Coffee	13 (29.5)	7 (22.6)	6 (20.0)			
Alcohol	16 (36.4)	15 (48.4)	15 (50.0)			
Cold, raw foods	15 (34.1)	10 (32.3)	8 (26.7)			
Spicy foods	17 (38.6)	6 (19.4)	8 (26.7)			
Others	10 (22.7)	3 (9.7)	1 (3.3)			

[a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between treated and untreated patients; [e]$P < 0.05$ by χ^2-test between IBS patients and controls.

Table 5 Skin prick test results using food allergens and inhalant allergens, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value [a]	*P* value [c]	*P* value [e]
Positive food SPT	17/44(38.6)	5/31 (16.1)	1/30 (3.3)	<0.001	0.03	<0.001
Saury	13 (76.5)	2 (40.0)	1 (100.0)			
Mackerel	3 (17.6)	-	-			
Beef	1 (5.9)	2 (40.0)	-			
Pork	2 (11.8)	1 (20.0)	-			
Buckwheat	3 (17.6)	-	-			
Rice	4 (23.5)	1 (20.0)	-			
Arrowroot	3 (17.6)	-	-			
Sweet potatoes	3 (17.6)	-	-			
Beans	2 (11.8)	1 (20.0)	-			
Cabbages	3 (17.6)	-	-			
Celery	3 (17.6)	-	-			
Onions	3 (17.6)	-	-			
Peach	1 (5.9)	1 (20.0)	-			
Tomato	1 (5.9)	-	-			
Melon	2 (11.8)	-	-			
Squid	1 (5.9)	1 (20.0)	-			
Trumpet shell	3 (17.6)	-	-			
Curry	2 (11.8)	-	-			
Positive inhalant SPT	11 (25.0)	11 (35.5)	13.3 (4)	NS	NS	NS
D-farinae	7 (63.6)	7 (63.6)	50.0 (2)			
D-pteronyssinus	5 (45.5)	8 (72.7)	50.0 (2)			
Alternaria alternate	2 (18.2)	-	25.0 (1)			
Grass pollen	27.3 (3)	1 (9.1)	-			
Tree pollen	36.4 (4)	1 (9.1)	1 (25.0)			
Mugwort pollen	-	2 (18.2)	-			
Willow pollen	9.1 (1)	-	-			
Ragweed	36.4 (4)	3 (27.3)	-			
Dog hair	45.5 (5)	1 (9.1)	1 (25.0)			
Cat fur	18.2 (2)	-	-			
Cockroach mix	9.1 (1)	-	-			

[a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between treated and untreated patients; [e]$P < 0.05$ by χ^2-test between IBS patients and controls.

resulting in food intolerance in the cause of gastrointestinal problems is much harder. Niec *et al*[18] reported that milk, wheat, eggs, potatoes, and celery are the most commonly identified factors causing gastrointestinal symptoms. Locke *et al*[19] reported that beans and legumes, chocolate, dairy products, and nuts are the most common foods causing hypersensitivity. It was reported that patients with IBS reveal intolerance to foods such as milk, bread, pizza, apple, hazelnut, tomato, egg, peach, and greens[7]. In our study, dairy products, coffee, alcohol, raw food, and spicy food were the most common foods causing problems. However, SPT was positive for saury, rice, mackerel, buck-

Table 6 IgE and total eosinophil count (mean ± SD)

	Treated (n = 44)	Untreated (n = 31)	Control (n = 30)	P value
IgE (IU/mL)	252.36±558.94	402.34±765.00	320.53±659.07	NS
Eosinophil (/μL)	153.74±149.68	181.72±126.04	166.42±139.10	NS

Table 7 Characteristics of treated and untreated patients with IBS, n (%)

Characteristics	Positive food SPT (n = 22)	Negative food SPT (n = 53)	P value
Gender			NS
Male	7 (31.8)	28 (52.8)	
Female	15 (68.2)	25 (47.2)	
Subtypes			NS
A-IBS	8 (36.4)	21 (39.6)	
C-IBS	9 (40.9)	13 (24.5)	
D-IBS	5 (22.7)	19 (35.8)	
Current history of allergies	13 (59.1)	33 (62.3)	NS
Reported food intolerance	9 (40.9)	7 (13.2)	0.013
Inhalant positive SPT	11 (50.0)	11 (20.8)	0.006
Positive RAST	3 (13.6)	-	

wheat, sweet potatoes, celery, onions, and trumpet shell. The results of different studies are inconsistent, which poses the question of the population specificity of such studies.

No correlation between SPT and patient's intolerance to certain foods was noted. There are several explanations for the discrepancies between reported food intolerance and SPT results. First, SPT is generally considered the most convenient and least expensive screening method for detecting allergic reactions in most patients. However, until the diagnostic efficacy of SPT is fully established with standardized allergens and methods, a positive skin test alone cannot confirm a definite clinical sensitivity to an allergen. Second, clinicians consider cross-reactions among various plants and animal allergens[20, 21]. The conservation of these proteins across biologic substances affects cross-reactivity in several ways. Certain foods (e.g., peanut) are able to sensitize and elicit reactions after oral exposure (type 1 allergy) and can trigger responses to related foods (e.g. legumes). Other foods (e.g., apples) containing labile proteins are not strong oral sensitizers. Helbling *et al*[22] have reported the clinical cross-reactivity to most fish species and several *in vitro* studies have demonstrated the existence of common allergens between different fish species[23-26]. The third explanation is that food allergies are localized at the intestinal mucosa and specific IgEs are primarily present in intestinal mucosa but not systemically. For this reason, Andre *et al*[27] have indicated the importance of stool IgE rather than serum IgE. Bischoff *et al*[28] have also stressed the importance of stool eosinophil counts and mast cell mediators.

Both SPT and RAST are for IgE-mediated disease, but there is no correlation between the two methods. However, RAST is not as sensitive as the skin test. As a result, in patients with a history of reactions to foods, insect stings, drugs, or latex, skin testing is still required because of its higher sensitivity even if the RAST is negative. The primary advantage of RAST over SPT is safety with the results not influenced by skin disease or medication[29].

In our study, the IBS patients showed much higher positive SPT rates than the controls ($P < 0.001$). The treated IBS patients were more likely to have a positive SPT than the untreated IBS patients ($P = 0.03$). The untreated IBS patients defined by fulfillment of the Rome IBS criteria complained of severe symptoms ($P < 0.05$) (Table 2), suggesting a possible relationship exists between IBS and allergic diathesis. Further studies are needed to explore this relationship. In addition, we also used common inhalant allergens to compare with the food allergens and did not find any difference in positive SPT between inhalant allergens among the three groups (25%, 35% and 13% respectively). White *et al*[30] reported that increased airway responsiveness to inhaled methacholine can be demonstrated in irritable bowel syndrome patients with no clinical evidence for atopic disease[31]. These findings are in contrast to the previous hypothesis that IBS is a generalized immune hypersensitivity state. These findings also suggest that food allergens may play a certain role in IBS patients. However, well designed dietary elimination and food challenge studies are needed to document the food hypersensitivity in IBS patients.

In this study, the untreated IBS patients were older than the treated patients ($P = 0.005$). Because age could affect SPT, it may confound our results. But the number and size of prick skin reactions increase throughout childhood until twenty years of age and then gradually decline until age fifty[32]. Even if age is considered, more treated patients were positive for SPT in our study.

In conclusion, more IBS patients are positive for SPT and food allergens than healthy controls. However, the IBS patients present more severe symptoms, lower overall quality of life, and higher positive SPT compared to the untreated IBS patients even though the history of allergies is not different.

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RAPID COMMUNICATION

Accuracy of combined PET/CT in image-guided interventions of liver lesions: An *ex-vivo* study

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Abstract

AIM: Positioning of interventional devices in liver lesions is a challenging task if only CT is available. We investigated the potential benefit of combined PET/CT images for localization of interventional devices in interventional liver studies.

METHODS: Thirty lesions each of hyperdense, isodense and hypodense attenuation compared to normal liver parenchyma were injected into 15 *ex-vivo* pig livers. All lesions were composed of the same amounts of gelatine containing 0.5 MBq of ^{18}F-FDG. Following lesion insertion, an interventional needle was placed in each lesion under CT-guidance solely. After that, a PET/CT study was performed. The localization of the needle within the lesion was assessed for CT alone and PET/CT and the root mean square (RMS) was calculated. Results were compared with macroscopic measurements after lesion dissection serving as the standard of reference.

RESULTS: In hypo- and isodense lesions PET/CT proved more accurate in defining the position of the interventional device when compared with CT alone. The mean RMS for CT and PET/CT differed significantly in isodense and hypodense lesions. No significant difference was found for hyperdense lesions.

CONCLUSION: Combined FDG-PET/CT imaging provides more accurate information than CT alone concerning the needle position in FDG-PET positive liver lesions. Therefore combined PET/CT might be potentially beneficial not only for localization of an interventional device, but may also be beneficial for guidance in interventional liver procedures.

INTRODUCTION

Treatment of liver lesions may be performed either surgically or interventionally. Surgery still represents the treatment of choice in solitary liver lesions or in patients with a limited number of lesions confined to a specific liver segment. However, certain cardiovascular or pulmonary risk factors may prevent patients from undergoing surgery with a curative intent [1,2]. In some patients minimally invasive interventional approaches may serve as an option for patient treatment. Interventional procedures for treatment of liver lesions include procedures such as radiofrequency ablation, laser induced ablation or chemoembolization [3-5]. Except for chemoembolization, all other therapeutic interventional procedures include placement of an interventional needle transcutaneously within the liver lesion. In most procedures, the needle is placed under CT, MRI, or ultrasound guidance. However, localization and guidance of an interventional device on morphological imaging can be challenging, particularly in selected cases where differentiation of the tumor from adjacent liver parenchyma may be difficult. These circumstances occur especially in tumors with only little or no contrast enhancement [6,7]. On the other hand, functional imaging has proved to be superior to morphological imaging in detection and characterization of these tumors, but is known for only limited anatomical information.

In any case accurate placement of an interventional device is indispensable for complete tumor treatment in every interventional method. In cases of equivocal findings on morphological imaging procedures, additional functional data can provide information about viable tumor parts. Combined PET/CT imaging systems provide accurate morphological and functional data sets within a single operation [8].

The aim of this study was to evaluate a potential benefit of combined PET/CT imaging concerning mor-

phology and complementary functional information when assessing the position of an interventional needle within an isolated liver without breathing. This might potentially improve the accuracy of localization of interventional devices compared with morphological imaging alone.

MATERIALS AND METHODS

Lesion model

Ninety artificial lesions, two hypodense, two isodense, and two hyperdense were placed in each of 15 *ex-vivo* pig livers by needle injection. The injected substances consisted of the following ingredients: Hypodense lesions: gelatine (9 g), sodium chloride (NaCl: 20 mL). Isodense lesions: gelatine (9 g), NaCl (20 mL), iodine-based contrast agent (0.2 mL/300 mg/mL Xenetix 300, Guerbet GmbH, Sulzbach, Germany). Hyperdense lesions: gelatine (9 g), NaCl (20 mL), iodine-based contrast agent (0.4 mL/300 mg/mL). Additionally 0.5 MBq ^{18}F in form of [^{18}F]-2-Fluoro-2-deoxy-D-glucose (FDG) were administered to all mixtures, thus 0.5 MBq FDG, 20 mL sodium chloride, 9 g of gelatine and the different amounts of contrast agents were used per lesion.

The concentration of FDG was chosen based on previous measurements of differences between tumor and liver background in 75 patients with liver malignancies. A ratio using the differences of Standard Uptake Values (SUV) between the tumor and the liver background was established. Based on this ratio, the amount of FDG was chosen to establish the same ratio between the artificial lesions and the liver background. Seventy-five consecutive patients with metastases of colorectal carcinoma, 25 patients with metastases of NSCLC (non small cell lung cancer) and 10 patients with hepatocellular carcinomas (HCC) were chosen from the local PACS. Patients with HCC were only partly PET-positive tumors or had only low values (SUV), but were chosen to define a broad spectrum of liver malignancies.

Lesion injection and interventional procedure

First, the artificial lesions were injected into the liver with a 7-gauge needle (Figure 1). After injection of the artificial lesion into the *ex-vivo* pig livers, the gelatine mixture hardened as it cooled. Lesion injection and the subsequent interventional device placement were carried out by different physicians. Thus, one physician injected the artificial lesions, while another physician inserted the biopsy needles in the following step.

Second, 18 gauge biopsy needles were inserted (by the second physician) in each liver, one needle in each lesion for a total of 6 needles per *ex-vivo* liver under strict CT-guidance. The needles were only inserted in lesions with favourable shape (round, oval or elliptic) to guarantee reproducible measurements. Lesions which partly dropped away through bile ducts and veins were excluded from the measurements. Additionally, before needle placement, each liver was covered by a thin, non-transparent sheet before needle placement to hide the injection sites.

CT and PET/CT imaging

CT and PET/CT imaging were performed with a Biograph™ system (Siemens Medical Solutions, Hoffman Estates, IL).

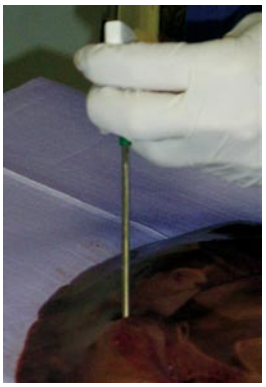

Figure 1 A 7-gauge biopsy needle and a syringe were used for insertion of the gelatine/FDG mixture into the liver. The mixture hardened as it cooled in the *ex-vivo*-liver.

The system consists of two components: a dual-slice CT scanner (Somatom Emotion, Siemens Medical Solutions, Erlangen, Germany) with a minimum gantry rotation time of 800 ms and a full ring PET tomograph (ECAT HR+, Siemens Medical Solutions, Hoffman Estates, IL). The PET system has an axial field of view of 15.5 cm per bed position and an in-plane spatial resolution of 4.6 mm. CT and PET images were acquired consecutively. Repetitive CT scans were carried out for needle positioning with a field-of-view focused on the interventional region. Acquisition parameters for the interventional CT were 120 mAs, 130 kV, 1 mm slice thickness and 1.5 mm table feed.

Following needle placement under strict CT guidance (without using any PET information) a combined PET/CT study was conducted with all needles in position covering the whole *ex-vivo* liver (CT: 120 mAs, 130 kV, 1 mm slice thickness with a 0.5 incremental reconstruction, 1.5 mm table feed, 1 mm collimation, PET: scan time 4 min, 3D data acquisition). CT and PET data sets from the combined imaging approach can be viewed separately or in fused mode on a commercially available computer workstation, which also allows distance measurements on fused images in all three dimensions. Thus, CT images and combined PET/CT images were evaluated separately while all needles inserted within the artificial lesions.

Image evaluation

CT images, either viewed alone or in fused mode (PET/CT), were adjusted to the soft tissue window (center: 50 Hounsfield Units (HU), width: 350 HU). PET images were adjusted to Full Width at Half Maximum (FWHM).

The lesions' width and height were measured in the coronal plane on the commercially available computer workstation and the length was measured in the same way using MPR (multi planar reformatting) in the sagittal direction. The accuracy of CT data alone and combined PET/CT data for determining the needles' position was assessed by measuring the distance (in the horizontal direction) of the needle tip to the lesions' margins on both imaging procedures (CT alone and combined PET/CT) with an electronic calliper in the coronal, transverse and sagittal planes. Imaging measurements from the workstation were correlated with macroscopic measurements after liver lesion dissection, which served as the standard of reference. For this purpose, the liver, with all needles still in place, was finely sliced to measure the distance from the needles' tip to the lesion's margins macroscopically.

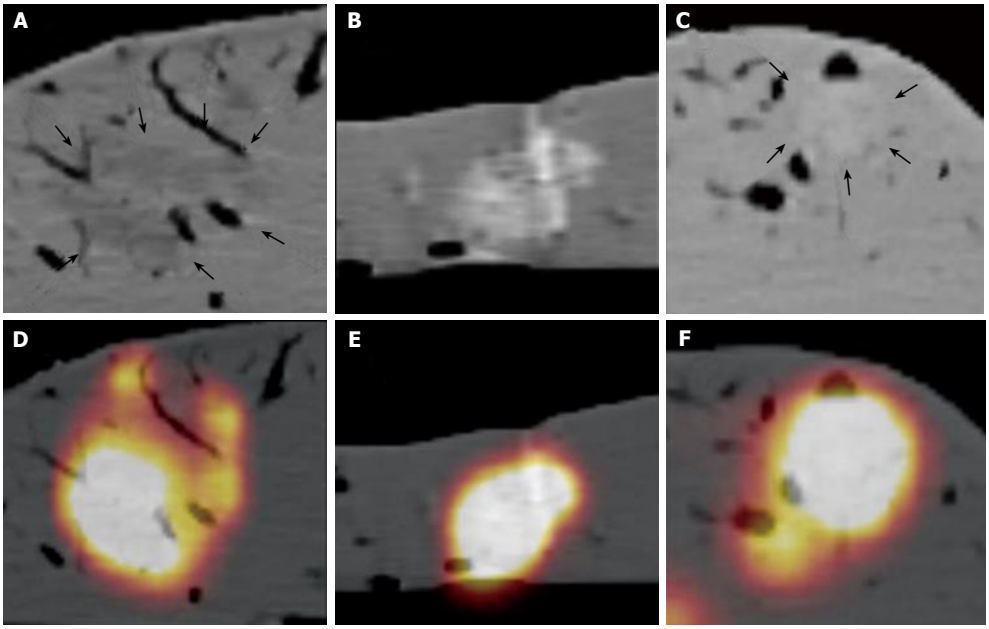

Figure 2 **A**: This shows a hypodense lesion. The margins of the hypodense lesion are barely seen (fine black arrows). In comparison, the combined PET/CT image of the same lesion shows exactly the margins of the lesion (Figure 2D). Furthermore, the combined PET/CT suggests a different location and extent of the lesion, which was confirmed by the standard of reference. **B**: This shows a hyperdense lesion. The margins of the lesions are well seen. The combined PET/CT shows the same location and extent of the lesion within the liver tissue (Figure 2E). Thus, in hyperdense lesions, a high correlation of CT and combined PET/CT measurements was found. **C**: This shows a nearly isodense lesion. The margins of the lesions can only be anticipated (fine black arrows). The corresponding PET/CT (Figure 2 F) of the same lesion shows a different extent of the same lesion with well defined and sharp margins.

All measurements concerning the position of the needle within a single lesion (distances to lesion margin) were compared separately, and the root mean square (RMS) was calculated. The RMS was chosen as a standard description to compare the mean values of different group measurements. The Root Mean Square is calculated as follows:

$$RMS = \sqrt{(x-x_0)^2+(y-y_0)^2+(z-z_0)^2}$$

In this formula, x, y and z are defined as the values derived from the macroscopic measurements, serving as the gold standard. In comparison, x_0, y_0 and z_0 are the values from CT measurement or PET/CT measurement. Therefore, in this formula, the values of the gold standard and the values of CT and PET/CT are already compared by calculating the deviation of measurements from CT or PET/CT compared with the macroscopic standard of re-ference. Thus, greater deviations (meaning less exact definition of the needles' tip on CT or combined PET/CT compared to the standard of reference) will be represented by higher RMS values. In comparison, lower RMS values are calculated based on less deviation of the needles' tip compared to the standard of reference. Values (mean values) were calculated for every lesion based on CT and PET/CT measurements.

Additionally, Hounsfield Units (HU) of every lesion and their surrounding liver tissue was measured and mean values for surrounding tissue and every lesion type were calculated to compare the lesions densities in relation to the liver background. Wilcoxon test for comparing two paired groups was used for statistical analysis.

RESULTS

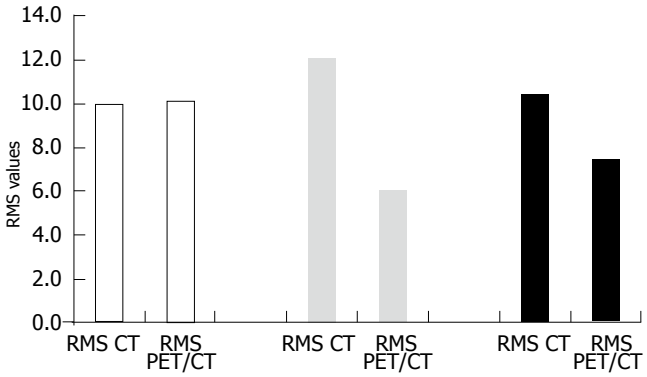

Figure 3 This shows the different mean RMS values for hyper-, iso-, and hypodens lesions. A statistically significant difference was found between the mean RMS values in CT and PET/CT in isodense lesions ($P < 0.05$, grey bars). Additionally, a statistically significant difference was found between the mean RMS values of CT and PET/CT in hypodense lesions ($P < 0.05$, black bars). No difference was found in hyperdense lesions (white bars).

In 90 lesions placed in 15 ex-vivo pig livers, 77 were left in favourable shape for needle intervention (Figure 2). Since several lesions dropped away through bile ducts and veins, 23 hyperdense, 28 isodense, and 26 hypodense lesions were left for measurement.

The mean width was 20.2 (standard deviation (SD): 9 mm) mm, length and height of these lesions were 18.6 (SD: 6 mm) mm, and 16 (SD: 5 mm) mm, respectively, according to the macroscopic standard of reference. Two isodense lesions were missed based on CT data due to poor visibility on the CT images only, whereas both lesions were clearly seen on combined PET/CT images only. The lesions were confirmed by liver dissection. There were no failed punctures in hypo- and hyperdense lesions. The mean RMS for the CT measurements and the combined

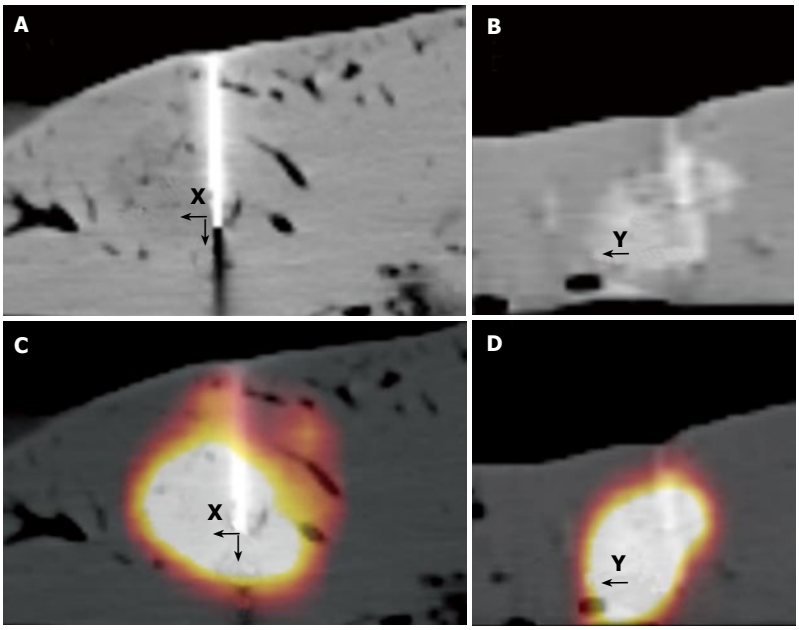

Figure 4 It shows the measurements on the commercially available computer workstation for CT alone (**A**/**B**) and combined PET/CT (**C**/**D**). The lesions´ width and height was measured in coronal direction (Figure 4**A** and **C**, X/Z), the lesions´ length in sagittal direction (black arrow (Y) in Figure 4 **B** and **D**).

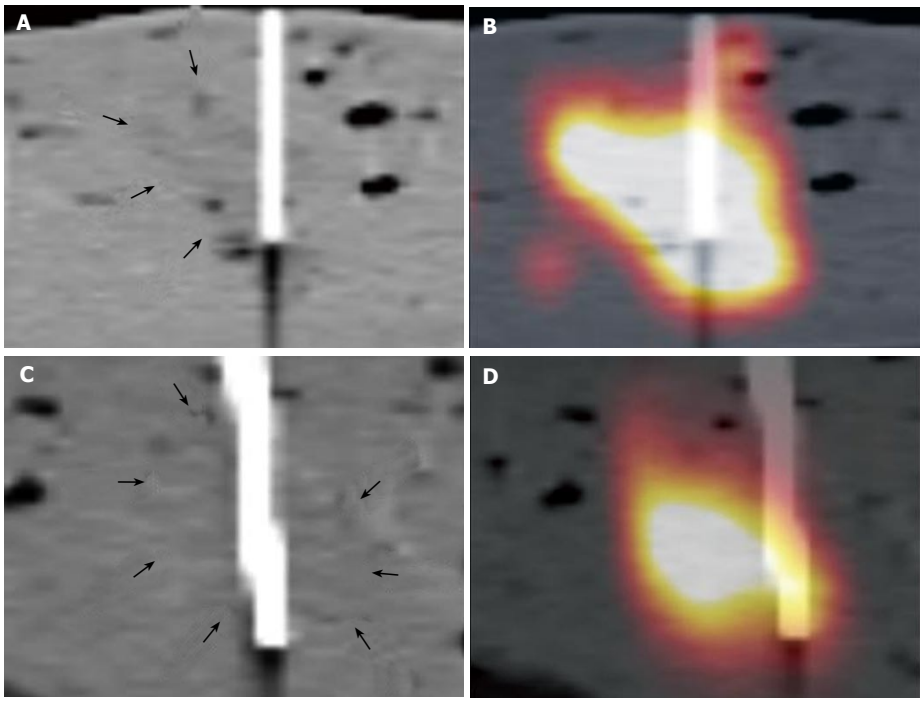

Figure 5 It shows an example of two isodense lesions on CT alone and combined PET/CT with the inserted biopsy needles. The lesions margins are barely seen on CT images alone (fine black arrows, **A** and **C**). In comparison, the true extent and localisation of the lesion are well shown on the combined PET/CT images. Based on poor visibility on CT alone, the lesion in the bottom row was nearly missed (**D**).

PET/CT in hyperdense lesions showed nearly identical values (mean RMS for CT: 10, mean RMS for PET/CT: 10.1) (Figure 3). On the other hand, significant differences were found for the comparison of isodense lesions (mean RMS for CT: 12.1, mean RMS for PET/CT: 6.1). The values for hypodense lesions were less different, however, a significant difference was found here as well (mean RMS for CT: 10.4, mean RMS for PET/CT: 7.4) (Figure 3). Differences between CT and PET/CT were statistically significant in hypodense and isodense lesions ($P < 0.05$ for hypo-, and isodense lesions). Thus, the definition of the needles' position within the lesion on combined PET/CT images was significantly more accurately defined than on CT images alone in hypodense and isodense lesions (Figure 4 and Figure 5). No statistically significant difference between CT-based and PET/CT-based measurements in hyperdense lesions was found. The distribution of mean Hounsfield Units (HU) for hypodense lesions, isodense lesions and hyperdense lesions, as well as for the surrounding liver tissue with corresponding standard deviation was measured and calculated as well (Figure 6).

The difference between mean values for hypodense lesions compared to the liver background was less than the difference of mean values of hyperdense lesions compared to liver background. Mean values of isodense lesions were only slightly different compared to mean values of the liver background, thus the model of artificial lesions met the requirements of the study.

DISCUSSION

PET/CT imaging proved more accurate when assessing

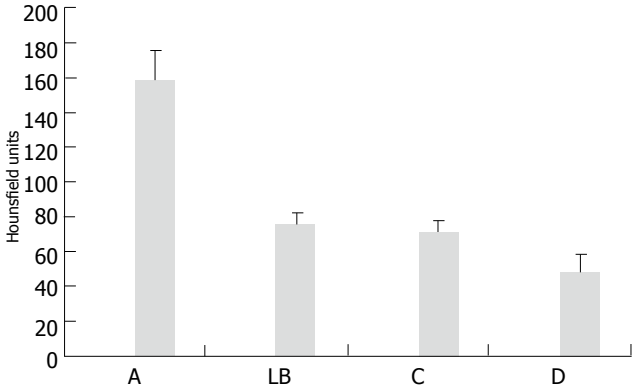

Figure 6 This shows the mean Hounsfield Units (HU) of all measured lesion types, the compared liver background and the corresponding standard deviation. **A**: hyperdense lesions; **LB**: liver background; **C**: isodense lesions; **D**: hypodense lesions.

the position of an interventional device within a liver lesion than imaging analysis based on morphological data alone. Hence, based on the option of more accurate detection of viable tumor tissue, PET/CT promises to add substantial information in selected liver interventions in which equivocal findings on morphologic imaging procedures complicate accurate determination of the interventional needle within the tumour.

Exact positioning of different interventional devices is indispensable for appropriate results in different interventional procedures. In cases of radiofrequency ablation (RFA), complete tumor necrosis is required, because inaccurate position of the ablation device will lead to much higher recurrence rate [9-11]. In biopsy procedures, however, differentiation of viable tumor tissue and necrosis might sometimes be difficult based on only slight differences in density. Furthermore, exact positioning may sometimes be problematic due to unfavourable shape, size, position and visibility of the lesions.

Although CT is a widely used tool for guidance of interventional procedures [12-14], even contrast-enhanced CT images may sometimes not be able to distinguish, both the margin of the lesion and the necrotic region from viable tumor fractions. Lesion attenuation values may differ after i.v. contrast enhancement depending on the tumour entity. Thus, metastases of colorectal cancer appear as hypodense lesions within a contrast enhanced liver, whereas HCC as a contrast-enhancing lesion appears with a higher attenuation than the surrounding liver tissue in the arterial phase. However, some lesions may not show lesion to background contrast after i.v. contrast enhancement, they appear isodense. Therefore a lesion model with different densities, representing several contrast enhancing patterns was chosen.

PET/CT provides additional data by adding functional information to morphology and may be of benefit in cases with impaired visibility and only partly viable lesions [15, 16]. In our study, the needles' positions were significantly better determined on combined PET/CT data sets in hypodense and isodense lesions than with CT alone. Two advantages may arise from these methods from the clinical point of view: first, the placement of the needle may be performed more accurately in cases with impaired visibility. Although PET/CT puncture itself was not performed in this study, these results are suggesting that the use of the additional functional information can possibly lead to the correct position of the ablative device within the lesion. Thus complete tumour ablation in RFA can be achieved. Second, the assessment of the positioning of interventional devices may be more accurate as well.

Needle positioning was significantly more accurate in hypodense and isodense lesion. While this result may be expected for isodense lesions, it seems rather surprising for hypodense lesions. After all, the needle position in hyperdense lesions was assessed equally well with CT alone and combined PET/CT. These results may be explained by attenuation values of the different lesions types. Differences between attenuation values of hypodense lesions and liver background were less than the difference between the mean Hounsfield Units of hyperdense lesions and liver background. Thus, lesion to background contrast was substantially higher for hyperdense lesions than for hypodense lesions (Figure 6). A further decrease in CT attenuation of hypodense lesions may lead to similar results as detected for hyperdense lesions in this study.

Additionally, two isodense lesions were missed by needle placement based on CT images alone. Primary puncture based on PET/CT images might presumably bring an advantage in visibility and might lead to a successful puncture in these cases.

The study has different limitations. Since it was conducted as an *ex-vivo* trial, patient-induced organ movement was excluded and unfavourable tumor sites close to the diaphragm or the liver hilum could be disregarded. Furthermore, this *ex-vivo* model did not include any background activity which may lead to an increased lesion detectability when assessing the needles' position on combined PET/CT. However, the concentration of FDG injected was chosen based on the described ratio calculated on measurements in patients with different liver malignancies. Thus, we tried to come close to a lesion to background ratio similar to those of patients with a broad average of liver malignancies.

Clinical disadvantages are the small size of the evaluated lesions less than 4 cm in diameter. However, small lesions are the ones most difficult to place an ablative device in. Thus, these are the ones in which CT or other morphological imaging procedures may fail. In addition, interventional procedures with a curative intent are limited to smaller lesions. For example, in RF-ablation the maximal diameter that may successfully be treated is 5 cm. Therefore, the lesion size chosen here met the requirements to simulate clinically often difficult procedures.

Additionally, effective treatment (especially in RFA) can particularly be delivered only to lesions with an appropriate, more or less round, shape. However, primary liver tumors for example can grow beside vessels and bile ducts. In these cases, other therapeutic options have to be considered. Since several lesions in our study setting drained away through bile ducts or veins, these unfavourable lesions were excluded from the evaluation as well.

A general consideration when implementing PET/CT-guided interventions in clinical practice will be its com-

plexity and additional cost compared to CT-guided liver interventions. This approach will be more time-consuming, based on patient preparation with FDG administration one hour prior to the intervention and a substantially longer scan time based on the PET component. Hence, this approach should be limited to cases in which CT alone is not able to accurately assess lesion size, shape and location. A possible clinical procedure when evaluating patients for (diagnostic or therapeutic) interventions could be to conduct a contrast enhanced CT scan first. If the target is well visualized and the lesions' margins are clearly defined on non-enhanced or contrast-enhanced images, a conventional CT-guided intervention might be performed. If the lesion is not clearly defined (hypodense or isodense), a separate PET/CT might be performed to identify the exact localisation and margins of the lesions. The combined PET/CT image then can possibly serve as a landmark tool for the CT-guided intervention. Another step further can be the direct, PET/CT-guided intervention. In this case, the interventional procedure has to be performed directly within the PET/CT system. However, the PET component will require, compared to CT alone, significantly more time, which presumably will be one limiting factor in this scenario. To date, there is no software available where the needle's position can be defined online under combined PET/CT-guidance. Hence, further studies with new technologies have to address the feasibility of this approach. In our opinion, this should be furthermore chosen with focus on cases in which a curative approach is aspired, based on the additional complexity, duration and costs of this procedure.

We conclude that combined PET/CT adds substantial additional information to CT alone when assessing the position of an interventional device within a liver lesion. Thus, co-registered PET/CT can be recommended to date for planning selected liver interventions but, based on its additional complexity and cost, should be limited to those procedures where CT alone is not able to accurately delineate the lesion in question.

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RAPID COMMUNICATION

Hepatocytic differentiation of mesenchymal stem cells in cocultures with fetal liver cells

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Abstract

AIM: To investigate the hepatocytic differentiation of mesenchymal stem cells (MSCs) in co-cultures with fetal liver cells (FLC) and the possibility to expand differentiated hepatocytic cells.

METHODS: MSCs were marked with green fluorescent protein (GFP) by retroviral gene transduction. Clonal marked MSCs were either cultured under liver stimulating conditions using fibronectin-coated culture dishes and medium supplemented with stem cell factor (SCF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor 4 (FGF-4) alone, or in presence of freshly isolated FLC. Cells in co-cultures were harvested, and GFP+ or GFP- cells were separated using fluorescence activated cell sorting. Reverse transcription-polymerase chain reaction (RT-PCR) for the liver specific markers cytokeratin-18 (CK-18), albumin, and alpha-fetoprotein (AFP) was performed in different cell populations.

RESULTS: Under the specified culture conditions, rat MSCs co-cultured with FLC expressed albumin, CK-18, and AFP-RNA over two weeks. At wk 3, MSCs lost hepatocytic gene expression, probably due to overgrowth of the cocultured FLC. FLC also showed a stable liver specific gene expression in the co-cultures and a very high growth potential.

CONCLUSION: The rat MSCs from bone marrow can differentiate hepatocytic cells in the presence of FLC *in vitro* and the presence of MSCs in co-cultures also provides a beneficial environment for expansion and differentiation of FLC.

INTRODUCTION

The existence of putative liver stem cells in the bone marrow was first described by Petersen *et al*[1] who showed that bone marrow cells transplanted into lethally irradiated mice could engraft in the recipient's liver and differentiate into hepatic stem cells (oval cells) or hepatocytes. First *in vitro* data suggest that several types of bone marrow cells / stem cells can differentiate towards hepatocytic cells under the appropriate culture conditions[2-5]. Oh *et al*[6] found that the liver specific proteins alpha-feto protein (AFP) and albumin are expressed in cultures of unsorted rat bone marrow cells after 21 d. Furthermore, several recent *in vitro* studies indicate the possibility of hepatocytic differentiation of MSCs *in vitro*[7-9]. Kang *et al*[7] reported that MSCs from the rat bone marrow can express the liver specific marker AFP, and produce albumin and urea *in vitro* when cultured in the presence of cytokines, fibroblast-growth factor (FGF-4) and hepatocyte growth factor (HGF). Hong *et al*[8] demonstrated that the liver specific genes cytokeratin (CK) 18, AFP, and albumin are expressed in cultures of human umbilical cord-blood derived MSCs. Furthermore, Lee *et al*[9] also showed liver specific functions of cytochrome P450 activity, urea and albumin-production, and glycogen storage in hepatocytic differentiated umbilical cord blood-derived MSC cultures. We had also shown that rat MSCs have a differentiation potential towards hepatocytic cells when co-cultured with adult rat liver cells[10,11]. The fetal milieu, however, might permit a more suitable environment for rapid MSC-maturation into hepatocytic cells.

In this study, we investigated the potential of rat mesenchymal stem cells derived from adult bone marrow to differentiate into hepatic cells *in vitro* under the direct influence of fetal liver cells for the initiation of liver specific gene expression.

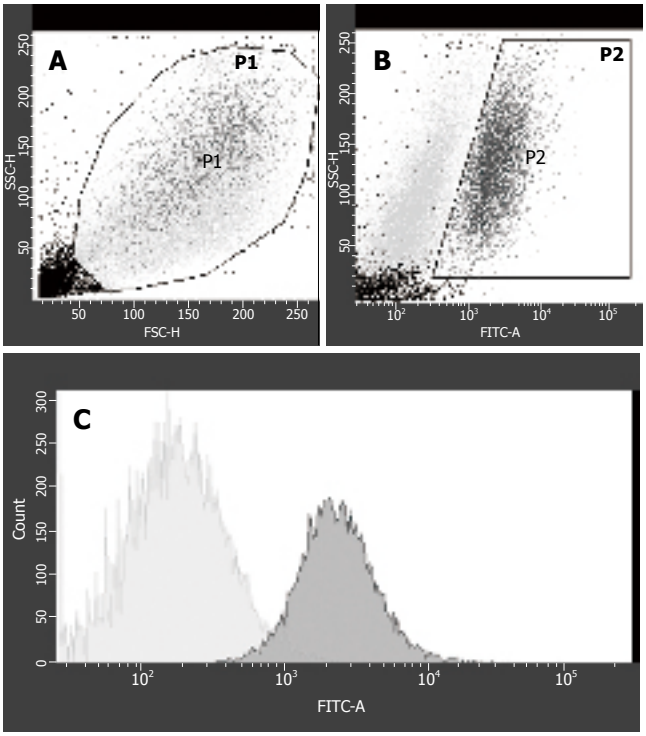

Figure 1 FACS sorting of GFP+ and GFP- cells of co-cultures at wk 1. **A**: Viable cells were gated as P1 cells; **B**: P1 cells were gated in GFP- or GFP+ (P2); **C**: For the highest purification of GFP+ cells, P2 cells were analyzed and only GFP+ cells were sorted for PCR-analysis (light grey peak), 34.5 % of cells from P2 were GFP+ and 65.5 % were GFP-.

MATERIALS AND METHODS

Rat mesenchymal stem cells and fetal liver cells

Isolation, transduction and cloning of rat mesenchymal stem cells were carried out as described previously[10]. Rat embryos of embryonal/ fetal day (ED) 16 were harvested by section after the mother was sacrificed by an overdose of ether-anesthesia. Fetuses were dissected under the dissection microscope. Fetal livers were harvested and liver cells were isolated by a collagenase digestion using collagenase type IV (Sigma, St. Louis, USA) as described previously[12]. A Percoll (P = 1 124 g/mL, Biochrom, Berlin, Germany) gradient centrifugation was used to enrich the viable cell fraction. The Percoll concentration used was 76%. Magnetic cell sorting was used for the removal of fetal OX-43 and OX-44 positive hematopoietic cells from the freshly isolated cells as described elsewhere[13]. In brief, isloated cells were marked with mAb OX-43 and mAb OX-44 (both Serotec, Eching, Germany). Unbound antibodies were removed and secondary marking was done with goat-anti-mouse IgG bound magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Positive cells were absorbed in a magnetic field by Vario-MACS columns, the depleted cell fraction was collected in a tube.

Co-culture of rMSCs with fetal rat liver cells

Cultures were performed according to our protocol published previously[10]. In brief, cells were seeded on culture wells coated with fibronectin (Sigma) on 24-well plates (Greiner). rMSCs (9×10^4) per well were seeded for MSC-controls, or 6×10^4 fetal liver cells per well for controls. For co-cultures of rMSCs with fetal rat liver cells, 9×10^4 rMSCs per well were preceded in 24-well plates for 2 - 3 h. Then, 6×10^4 fetal rat liver cells per well were added to the cultures. Medium was changed twice a week. Stem Span SFEM (Stem Cell Technologies, St. Katherinen, Germany) medium was supplemented with penicillin/streptomycin (Gibco), dexamethasone (Sigma), 100 ng/mL human recombinant stem cell factor (SCF), 20 ng/mL hepatocyte growth factor (HGF) (both Immunotools, Friesoythe, Germany), 50 ng/mL epidermal growth factor (EGF), and 10 ng/mL fibroblast growth factor-4 (FGF-4) (both R&D, Wiesbaden, Germany). Cultures were analyzed on d 7, 14, and 21.

Separation of GFP positive and negative cells in co-cultures by fluorescence activated cell sorting (FACS)

After the culture period, cells from co-cultures were trypsinized, counted with trypan blue, and re-suspended in 3mL PBS. To get single cell suspension, cells were filtered through a 35 μm filter (BD, Heidelberg, Germany). Cells were sorted using the FACS Aria™ Cell Sorter (BD) into GFP-positive (GFP+) or GFP-negative (GFP-) cells, focussing on the highest possible purity of GFP+ cells.

RNA extraction from cells by RT-PCR

RNA was extracted using the Invisorb Spin Cell-RNA™ Mini-kit (Invitek, Berlin, Germany) according to the manufacturer's instructions and stored at -80℃. Reverse transcription (RT) of extracted RNA was performed using the bulk first-strand c-DNA synthesis kit (Amersham, Freiburg, Germany). The cDNA was stored at -20℃. For PCR, 5 μL of cDNA-template was mixed with 2.5 μL of 10× PCR-buffer, 0.5 μL of 10 mmol dNTP´s, 0.5 μL of each primer (50 ng/μL), and 0.5 μL of polymerase (AmpliTaq., Gibco) in a total volume of 25 μL for each probe. PCR was carried out in a programmable Biometra Uno-Thermobloc (Biometra, Göttingen, Germany) using the primers for albumin, AFP, and CK-18 as described previously[10]. Negative controls were performed for each set of primers. Samples were analyzed on 1% agarose gels. The size of the PCR-fragments was estimated using the 100-base-pair ladder (Gibco BRL).

RESULTS

FACS of co-cultured cells

The sorting strategy concentrated on maximal purity of GFP+ cells to exclude contamination with FLC-derived RNA. A typical example for FACS 1 wk after co-culture is shown in Figure 1. The viable cells were gated according to forward and side scatter properties (Figure 1A). Two populations varied in size and granulation. GFP-expression of the gated cells was investigated in two populations, differing in granulation (Figure 1B) and gated as P2 cells. From these P2-cells, the contaminating GFP- cells were sorted out (Figure 1C). All non-P1, non-P2 and non-GFP cells were collected as GFP- cells. When the number of viable cells (gate P1) was set as 100%, the fraction of GFP positive cells decreased from 24.2 % of viable cells at wk 1 to 1.3 % of viable cells at wk 3 (Table 1). This was also con-

Table 1 Fractions of FAC-sorted cells (%)

	Wk 1	Wk 2	Wk 3
Viable Cells	70.2	79.1	77
GFP-positive cells	34.5	5.6	1.7
GFP-negative cells	65.5	94.4	98.3

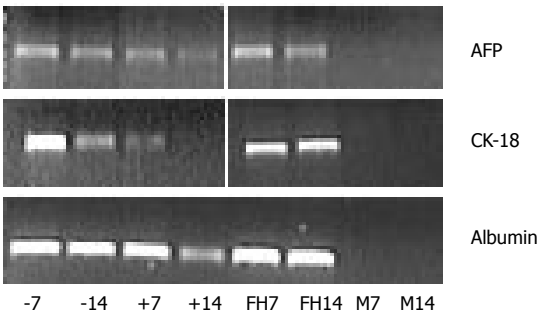

Figure 2 Expressions of the liver specific markers (AFP, CK-18, or albumin) of cultured rMSCs (M), fetal liver cells (FH), and GFP+ (+) or GFP- (-) cells of the co-cultures on d 7 or 14. Cultured rMSCs lacked expression of AFP, CK-18, or albumin on d 7 or 14 (lanes M7 and M14). Cultured fetal liver cells showed expression of AFP, CK-18 and albumin on d 7 or 14 (lanes FH7 and FH14). The GFP+ cells (lanes +7 and +14) showed clear expression of albumin and AFP, and lower expression of CK-18. The GFP- cells (lanes -7 and -14) showed a stable expression of AFP, CK-18, and albumin.

sistent with the morphological findings in the co-cultures, where a massive overgrowth of the adherent MSCs to the fetal liver cells was observed.

Liver specific geneexpression (AFP, CK-18, albumin)

Glyceraldehyde phosphate dehydrogenase (GAPDH) expression in cultured rMSCs, fetal liver cells, or GFP+ and GFP- FACS cells was equal at all time points (data not shown). Cultured rMSCs showed no expression of AFP, CK-18, or albumin on d 7 or 14 (Figure 2, lanes M7 and M14). Cultured fetal liver cells showed expression of AFP, CK-18 and albumin over the two wk culture period (Figure 2, lanes FH7 and FH14). The GFP+ cells (Figure 2, lanes +7 and +14) showed a strong expression of albumin and AFP, and a declining expression of CK-18. The GFP- cells (Figure 2, lanes -7 and -14) showed a stable expression of AFP, CK-18, and albumin. Negative controls without template were negative at all time points and probes (data not shown). On d 21, no liver specific genes were detected in the GFP+ cells (data not shown) probably due to the small cell number.

DISCUSSION

In this study, cloned GFP+ rMSCs from passage ⩾9 were used for the differentiation analysis towards liver cells. For liver specific differentiation, cells were cultured on a fibronectin matrix in serum free medium containing the liver growth factors: HGF, EGF, FGF-4, and the stem cell growth factor: SCF. The impact of FLC on hepatic differentiation was assessed in co-cultures of GFP+ rMSCs with freshly isolated fetal rat liver cells (GFP-negative), and compared to cultures with rMSCs or FLC alone. For gene-expression analysis, cells from co-cultures were separated in GFP+ or GFP- cells by fluorescence-activated cell sorting before RT-PCR analysis.

Albumin is a typical marker of mature hepatocytes, whereas CK18 is expressed in several liver cell types, including biliary epithelial cells and hepatic oval cells[14]. AFP in the liver is a marker of immature (e.g. fetal) liver cells or oval cells in the adult liver[15]. Our data indicate that rMSCs possess a differentiation potential towards hepatocytic cells *in vitro*. Expression of the liver specific genes CK-18, albumin, and AFP was demonstrated in GFP+ cells of the cocultures for two weeks. Additionally, we found that liver specific gene expression in mesenchymal stem cells was induced by the coculture with isolated FLC. Cultured rMSCs alone did not express any of the liver specific genes studied in the presence of fibronectin (FN)-coating and liver-differentiation stimulating growth factors. Avital *et al*[16] have highlighted the effects of co-cultured hepatocytes (separated by a PTFE-membrane) for β2m-negative thy1-positive stem cells from rat bone marrow expressing the liver marker albumin[16]. An important influence of hepatocytes on the differentiation of stem cell-enriched bone marrow has also been highlighted by Okumoto *et al*[17] who found that MSCs-enriched bone marrow stem cells cultured in the presence of HGF and fetal bovine serum (FBS) express the markers: hepatic nuclear factor 1 (HNF1-α) and CK-8 only after 7 d. In co-cultures with hepatocytes separated by a semi-permeable membrane the stem cells additionally express the liver specific markers: AFP and albumin. We found a beneficial effect for differentiation and growth of the co-cultured fetal liver cells (GFP-), which also showed a stable liver specific gene expression and viability over the whole observation period. This is consistent with findings of Hoppo *et al*[18] who showed a positive influence of MSCs on cultured endodermal fetal liver cells of the mouse in culture. However, the FLC tended to overgrow the adherent layer of MSCs at the end of the culture period. Thus, we could not detect any liver specific gene expression in the few remaining GFP+ MSC-derived cells in the co-cultures at wk 3. Our results support the notion of a faster differentiation of MSCs into hepatocytic cells under the influence of fetal liver cells compared to adult hepatocytes. The growth potential of MSCs seems to be impaired by the huge overgrowth of FLC, thus limiting the desired expansion of differentiated MSCs. Based on the declined GFP-positive fraction of cells and the decreased intensity of semi-quantitative PCR-signals over the experimental time of 3 wk, differentiation of rMSCs into hepatocytic cells could be suggested. However, fusion events can not be ruled out by our data.

Our *in vitro* data indicate that mesenchymal stem cells from rat bone marrow possess a differentiation capacity towards hepatocytic cells *in vitro*. Furthermore, we showed a strong influence of co-cultures with fetal liver cells on the induction of liver specific gene expression of cultured stem cells. However, fetal liver cells tended to overgrow the adherent MSCs in the co-cultures.

In conclusion, the co-culture of MSCs and FLC is a feasible culture model which might provide more insights into the relationship between fetal hepatopoietic cells during liver development.

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RAPID COMMUNICATION

Does *Helicobacter pylori* infection eradication modify peptic ulcer prevalence? A 10 years' endoscopical survey

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Abstract

AIM: To compare peptic ulcer prevalence in patients referred for upper gastrointestinal endoscopy in two Italian hospitals in pre-*Helicobacter* era and ten years after the progressive diffusion of eradication therapy.

METHODS: We checked all the endoscopic examinations consecutively performed in the Gastroenterology Unit of Padova during 1986-1987 and 1995-1996, and in the Gastroenterology Unit of Parma during 1992 and 2002. Chi Square test was used for statistic analysis.

RESULTS: Data from both the endoscopic centers showed a statistically significant decrease in the prevalence of ulcers: from 12.7% to 6.3% ($P < 0.001$) in Padova and from 15.6% to 12% ($P < 0.001$) in Parma. The decrease was significant both for duodenal (from 8.8% to 4.8%, $P < 0.001$) and gastric ulcer (3.9% to 1.5%, $P < 0.001$) in Padova, and only for duodenal ulcer in Parma (9.2% to 6.1%, $P < 0.001$; gastric ulcer: 6.3% to 5.8%, NS).

CONCLUSION: Ten years of extensive *Helicobacter pylori* (*H pylori*) eradication in symptomatic patients led to a significant reduction in peptic ulcer prevalence. This reduction was particularly evident in Padova, where a project for the sensibilization of *H pylori* eradication among general practioners was carried out between 1990 and 1992. Should our hypothesis be true, *H pylori* eradication might in the future lead to peptic ulcer as a rare endoscopic finding.

Key words: Ulcer prevalence; *H pylori*

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INTRODUCTION

It is now well established that *Helicobacter pylori* (*H pylori*) eradication can significantly modify the natural history of peptic ulcer disease. Marshall *et al* first demonstrated in 1988 that when *H pylori* was cleared 92% of ulcers healed and only 21% relapsed during a 12 mo follow-up period[1]. Several studies have then confirmed these data over the years. Follow-up studies have in fact shown lower relapse rates of both gastric and duodenal ulcers after successful *H pylori* eradication, in the short term as in the long term[2-4]. So by the end of the eighties *H pylori* was generally accepted as a causal factor in the pathogenesis of chronic gastritis and peptic ulcer. Between the end of the eighties and the beginning of the nineties eradication of *H pylori* has become a widespread approach for acid-related disorders, first among GI specialists, then among general practitioners.

Leerdam *et al* (2003) described a reduction in the incidence of upper gastrointestinal bleeding between 1993/94 and 2000[5]. They hypothesized that the decrease in incidence might partly be explained by the fact that *H pylori* is more often eradicated in patients with dyspeptic complaints and peptic ulcer disease, thus reducing the possibility of development of complications as bleeding. Furthermore, it is often reported as an impression by endoscopists that we see fewer ulcers than we did years ago, but data on the real prevalence are still scanty. Xia *et al* (2001) studied the prevalence of *H pylori* infection, peptic ulcer disease and reflux esophagitis in consecutive patients referred for upper GI endoscopy in an endoscopy unit in Sydney in a three-month period in different years (1990, 1994 and 1998), reporting a decrease in peptic ulcer disease

(22%, 15% and 13%, respectively, $P = 0.003$). They also described a lower prevalence of *H pylori* infection as well as a significant decrease in NSAIDs consumption, leading to the hypothesis that both these two risk factors likely contributed to the reduction of peptic ulcer disease[6].

Therefore, the aim of this study was to compare the prevalence of peptic ulcer disease among patients referred for upper GI endoscopy between the eighties and the nineties and after a period of 10 years, in two Italian GI units of two hospitals.

MATERIALS AND METHODS

Patients

We retrospectively analyzed all the upper GI endoscopies performed in the Gastroenterology Unit of Parma in two different years (1992 and 2002), and in the Gastroenterology Unit of Padova in two periods: from Feb 1, 1986 to Dec 31, 1987 and from Feb 1, 1995 to Dec 31, 1996. We selected the patients with a diagnosis of gastric or duodenal ulcer. Both the endoscopic units of Parma and Padova serve in-patients and out-patients, and are the major endoscopy centers of the area. Out-patients are directly sent by general practitioners or by specialists. Both the units have an informatic database (DB3 engine). We searched for gastric and duodenal ulcers both manually and informatically, with the strings "(gastrica or gastriche or angolare or antrale or del corpo-fondo) and/or (duodenale/i or bulbare or bulbari or del duodeno) and (ulcera or ulcere)".

Between 1990 and 1992 a project was performed in Padova in order to stimulate the aptitude towards the *Helicobacter pylori* (*H pylori*) eradication among physicians. It involved both specialists and general practitioners.

H pylori status was obtained for all patients accessing the hospital, through the dosage of serum antibodies. Data about upper GI symptoms and history of acid-related disorders were also collected. All patients positive for *H pylori* infection were treated with a triple one week therapy ("ulcer-free hospital" project).

Several meetings were held by the Gastroenterology Clinic of Padova between 1990 and 1992, with the participation of groups of 30 general practitioners. Statements discussion and interactive clinical case analysis were conducted, with initial and final testing of *H pylori* . General practitioners were sensibilized to test all patients suffering from upper GI symptoms or with a history of gastritis or peptic ulcer, diagnosed with a structured questionnaire ("ulcer-free ambulatory") and eradicate *H pylori*, when positive.

Statistical analysis

Chi Square test was applied on the changes of prevalence of peptic ulcers (total), gastric and duodenal ulcers after a decade. $P < 0.05$ was considered as significant.

RESULTS

In Parma we analyzed a population of 3779 subjects in 1992 (2185 out-patients and 1594 in-patients), with a mean age of 69.4 years (range 5-94 years) and a sex distribution of 54.4% males and 45.6% females, as well as a population of 3828 subjects in 2002 (1985 out-patients and 1843 in-patients), with a mean age of 62.3 years (range 12-97 years) and a sex distribution of 53.4% males and 46.6% females. We found 588 ulcers in 1992 (239 GU and 349 DU), 459 ulcers in 2002 (223 GU and 236 DU). *H pylori* status was evaluable only for 28.7% of patients with peptic ulcer in 1992 and 47.7% in 2002, so it could not be useful for statistical analysis. Among gastric ulcers, we found neoplastic lesions in 56 subjects in 1992 and 20 subjects in 2002.

In Padova we analyzed a population of 3703 subjects during 1986-1987, with a mean age of 54 years (range 15-91 years) and a sex distribution of 53.5% males and 46.5 females, as well as a population of 5727 subjects during 1995-1996, with a mean age of 51 years (range 14-98 years) and a sex distribution of 39.7% males and 60.3% females. We found 470 ulcers during 1986-87 (144 GU and 326 DU), 361 ulcers during 1995-1996 (86 GU and 275 DU). *H pylori* status was available only for patients with peptic ulcer during 1995-1996 and showed a prevalence of 83.6% (68.6% for GU and 88.4% for DU). It was determined by histology of mucosa, with appropriate staining.

Table 1 and Table 2 summarize the changes in prevalence of peptic ulcer after ten years in Parma and in Padova. Table 3 and Table 4 describe the epidemiological characteristics of the populations we studied.

Both the endoscopic centers showed a statistically significant decrease in the prevalence of ulcers: 12.7% to 6.3% ($P < 0.001$) in Padova, 15.6% to 12% ($P < 0.001$) in Parma. The decrease was greater for duodenal ulcer (8.8% to 4.8%, $P < 0.001$ in Padova, 9.2% to 6.1%, $P < 0.001$ in Parma) than for gastric ulcer (3.9% to 1.5%, $P < 0.001$ in Padova, 6.3% to 5.8%, NS in Parma).

Table 1 Changes in prevalence of peptic ulcer after ten years in Padova

Padova	1986-1987 *n* (%)	1995-1996 *n* (%)	*P*
Population	3703	5727	
Total of ulcers	470 (12.7)	361 (6.3)	< 0.001
Duodenal ulcer	326 (8.8)	275 (4.8)	< 0.001
Gastric ulcer	144 (3.9)	86 (1.5)	< 0.001

Table 2 Changes in prevalence of peptic ulcer after ten years in Parma

Parma	1992 *n* (%)	2002 *n* (%)	*P*
Population	3779	3828	
Total of ulcers	588 (15.6)	459 (12)	< 0.001
Duodenal ulcer	349 (9.2)	236 (6.1)	< 0.001
Gastric ulcer	239 (6.3)	223 (5.8)	NS

Table 3 Characteristics of the population from Parma

	1992	2002
Population (*n*)	3779	3828
Origin	2185 out-patients, 1594 in-patients	1985 out-patients, 1843 in-patients
Age (yr)	69.4 (range 5-94)	62.3 (range 12-97)
Sex (M/F)	54.4%/45.6%	53.4%/46.6%

Table 4 Characteristics of the population from Padova

	1986-1987	1995-1996
Population (*n*)	3703	5727
Age (yr)	54 (range 15-91)	51 (range 14-98)
Sex (M/F)	39.7%/ 60.3%	53.5%/ 46.5%

DISCUSSION

The study suggests that the incidence of peptic ulcer among patients referred for upper GI endoscopy significantly decreased through the years. Our hypothesis is that *H pylori* eradication could have changed the natural history of peptic disease. Eradication of the bacterium by the general practitioners in symptomatic subjects has become a common approach through the nineties. So we identified those patients who have not been eradicated or who were still symptomatic after the therapy.

One limit of the present study is the examination of a selected population, which was referred for upper GI endoscopy and probably had been given antisecretory drugs during the weeks preceding the access to the endoscopy. We have asked ourselves if the reduced prevalence of ulcer after ten years could be related with differences in drug prescription of anti-secretive agents among general practitioners. We did not collect data on drug intake, however, we thought this finding might only play a secondary role in the observed trend. In fact, significant differences in drug prescription could be seen when comparing the early eighties to the nineties, and since the late eighties histamine H2-receptor antagonists (anti-H2s) and proton pump inhibitors (PPIs) have been widely used in both the Italian areas we examined.

Capurso *et al* (1996)[7] retrospectively analysed upper gastrointestinal endoscopies performed in their center in Rome between January 1981 and December 1991. They reported an incidence of 4.1%±0.6% and a mean annual prevalence of 6.9%±0.7%. These data are quite similar to those in our center at the beginning of the observation period.

Data in Parma for peptic ulcer prevalence are quite similar to those reported by Xia *et al* in Sidney during the same decade. The authors concluded that both the decreased use of NSAIDs and the decline of *H pylori* infection have likely contributed to the reduction of peptic ulcer disease. Regretfully we did not have data about the *H pylori* status and the NSAIDs use of the population we examined. A reduced use of NSAIDs during the last decade has, however, not been reported in our areas, so we do not think it could have played a significant role.

The two populations examined in the present study showed different prevalence of both duodenal and gastric ulcer. We are not sure if this reflected a really different prevalence in the general populations of Parma and Padova, since no available data were collected on this subject. On the other hand, it must be underlined that the organization of both endoscopic units was similar; they tested both in-patients and out-patients, directly sent by general practitioners or by specialists.

Padova showed a greater significant decrease in the prevalence of ulcers through the decade. This might be due to the fact that the Gastroenterology Department of the University of Padova performed during those years a diffuse sensibilization of general practitioners about the eradication of *H pylori*, by means of the so called "Ulcer Free Project", as above described.

The decrease in prevalence was greater for duodenal ulcer than for gastric ulcer in both the studied populations. This is probably related to the different role played by *H pylori* in gastric and duodenal ulcer pathogenesis: it is known that more than 90% of duodenal ulcers but only 70% of gastric ulcers are associated with *H pylori* infection. This is in line with the results of the meta-analysis conducted by Ford *et al*[8]. They showed a reduction of relative risk of 54% in the recurrence of duodenal ulcer after *H pylori* eradication, and a still significant but smaller reduction of relative risk of 37% for gastric ulcer.

Additionally, it must be stressed that the role of anti-inflammatory drugs in the pathogenesis of gastric ulcer could be important in trying to correctly explain these data, but as previously mentioned, we lack at present epidemiological data on this subject.

In conclusion, we think *H pylori* eradication may in the future lead to peptic ulcer as a rare endoscopic finding, particularly in areas where a diffuse information program among general practitioners is performed. By now, the absolute number of ulcers we have diagnosed is still high, and there is need for more prevention strategies.

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RAPID COMMUNICATION

Conventional colonoscopy and magnified chromoendoscopy for the endoscopic histological prediction of diminutive colorectal polyps: A single operator study

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Abstract

AIM: To accurately differentiate the adenomatous from the non-adenomatous polyps by colonoscopy.

METHODS: All lesions detected by colonoscopy were first diagnosed using the conventional view followed by chromoendoscopy with magnification. The diagnosis at each step was recorded consecutively. All polyps were completely removed endoscopically for histological evaluation. The accuracy rate of each type of endoscopic diagnosis was evaluated, using histological findings as gold standard.

RESULTS: A total of 240 lesions were identified, of which 158 (65.8%) were non-neoplastic and 82 (34.2%) were adenomatous. The overall diagnostic accuracy of conventional view, and chromoendoscopy with magnification was 76.3% (183/240) and 95.4% (229/240), respectively ($P < 0.001$).

CONCLUSION: The combination of colonoscopy and magnified chromoendoscopy is the most reliable non-biopsy method for distinguishing the non-neoplastic from the neoplastic lesions.

Key words: Colorectal polyps; Colonoscopy; Chromoendoscopy; Magnifying endoscopy

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INTRODUCTION

Recently, colorectal polyps have been identified with increasing frequency as a result of the wide use of colonoscopy for screening. The detection and subsequent removal of neoplastic colorectal lesions, including adenomatous polyps and early cancers can reduce the incidence and mortality of colorectal cancers. Although initial data suggest that 80%-90% of diminutive colorectal polyps (≤5 mm) are histologically hyperplastic, more recent data indicate that 40%-60% of such polyps are neoplastic [1-5]. The ability to accurately differentiate the adenomatous from the non-adenomatous polyps is useful because it obviates the need for biopsies during colonoscopy and for removal of all diminutive polyps[6-8]. Some investigators suggest that the combination of magnified chromoendoscopy and colonoscopy can make the histopathological diagnosis of colorectal polyps. However, how this technique increases the accuracy of differential diagnosis of adenomatous from non-adenomatous polyps over conventional colonoscopy is unclear.

This study was to compare the observations by conventional colonoscopy to these obtained by magnified chromoendoscopy in a group of patients with diminutive colorectal polyps (≤5 mm), using histopathology as a "gold standard".

MATERIALS AND METHODS

Patients and exclusion criteria

Patients were eligible to participate in the study if they were scheduled to undergo flexible colonoscopy in our center. Patients with polyps larger than 5 mm were excluded from this study. Patients with inflammatory bowel disease (IBD), hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) were also excluded.

The study protocol was approved by the institutional review board of our hospital. An informed consent was

Table 1 Characteristics of patients and indications for chromoendoscopy

Characteristics of patients	
Patients (*n*)	150
Gender (males/females)	93/57
Age (yr), mean (range)	58.9 (37-85)
Indications for chromoendoscopy	
Screening	58
Family history	50
Lower GI bleeding	26
Altered bowel habit	16

Table 2 Characteristics and distribution of colonic lesions

No. of lesions	240
0-Ip (protruded, pedunculated)	18
0-Is (protruded, sessile)	122
0-IIa (superficial, elevated)	100
Adenomatous polyps	82
Non-adenomatous polyps	158
Distribution of colonic lesions	
Rectum	76
Sigmoid	96
Left colon	22
Transverse	6
Right colon	40

Table 3 Actual and predicted histology of 240 polyps by conventional colonoscopy

	Histology	
	AP[1]	non-AP[1]
Endoscopic prediction: AP	75/82	50/158
Endoscopic prediction: non-AP	7/82	108/158

[1]AP = adenomatous polyps; non-AP = non-adenomatous polyps.

obtained from each patient for the inclusion in the study.

Diagnostic procedures

A zoom-colonoscope (Olympus CF-Q160ZL, Olympus Optical Co., Ltd., Tokyo, Japan) providing both conventional and magnified images was used. The colonoscope was introduced up to the cecum. Subsequently, while the instrument was retracted, the colorectal mucosa was scrutinized in detail. When a polyp was detected, the mucus on the surface of the lesion was washed away and the polyp was first examined with the conventional view. Then 0.5% indigo carmine dye was sprayed over the lesion with a special catheter and observation was carried out. On conventional view, the key endoscopic findings for distinguishing non-neoplastic from neoplastic lesions were based on the gross appearance at visual inspection, namely the size, shape, overlying mucus, and color of the lesions. Macroscopic appearance was reported according to the Paris endoscopic classification of superficial neoplastic lesions[9].

The polyps were categorized as neoplastic or non-neoplastic as previously described[10]. The diagnosis at each step was recorded consecutively and all polyps were completely removed endoscopically. The accuracy rate of each type of endoscopic diagnosis was evaluated, using histological findings as gold standard. Morphology, size, and anatomical location of all lesions were documented. The diameter of each lesion was estimated using standard fully open biopsy forceps (4 mm) with the height estimated by placing the closed forceps tip adjacent to the lesion (2 mm). A single colonoscopist who was well trained in magnified colonoscopy, performed all procedures.

Statistical analysis

The number of patients in each group to demonstrate statistical significance with 5% alpha error and 90% power was estimated to be 47 and calculated on the basis of previous data[11-14], assuming a 25% difference in negative predictive value (NPV) for adenomatous polyps between the two groups. A chi-square analysis was performed for comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Of the 1982 consecutive patients attending for routine colonoscopy, 150 (93 men and 57 women with a mean age of 58.9 years) with polyps smaller than 5 mm in diameter were recruited between December 2004 and July 2005. Characteristics of the patients enrolled and indications for endoscopy are shown in Table 1. Total colonoscopy and histological evaluation were carried out in all patients without complications. A total of 240 lesions were identified. A single lesion was found in 101 (67.3%) cases, and 35 (23.3%) and 14 (9.3%) patients had two or more than three lesions, respectively.

A total of 158 lesions (65.8%) were non-neoplastic (including hyperplastic polyps, inflammatory polyps, and lymphoid aggregates) and 82 (34.2%) were adenomatous. Of these lesions, 194 (80.8%) were located in the distal colon (below the splenic flexure) and 46 (19.2%) in the proximal colon. The mean size of the non-neoplastic and neoplastic lesions was 3.46 mm and 3.83 mm, respectively. Although the neoplastic lesions might be larger than the non-neoplastic ones, the difference was not significant. The characteristics and distribution of colonic lesions are shown in Table 2.

The surface pattern of the lesion could be obtained and interpreted adequately in all cases. The overall diagnostic accuracy for distinguishing the neoplastic from the non-neoplastic lesions at each step (that is, conventional colonoscopy and magnified chromoendoscopy) was 76.3% (183/240), and 95.4% (229/240), respectively. The method of magnified chromoendoscopy with indigo carmine dye was significantly better for distinguishing the neoplastic from the non-neoplastic lesions, compared with conventional colonoscopy ($P < 0.0001$).

Fifty-seven of 240 lesions were misdiagnosed by conventional colonoscopy, including 38 hyperplastic polyps, 8 inflammatory polyps and 4 lymphoid aggregates which were overdiagnosed and 7 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, conventional colonoscopy had a sensitivity of 91.4%, specificity of 68.3%, NPV of 93.9% and PPV of 60.0% (Table 3). Eleven of 240 lesions were misdiagnosed by magnified chromo-

Table 4 Actual and predicted hystology of 240 polyps by high-resolution chromoendoscopy

	Histology	
	AP[1]	non-AP[1]
Endoscopic prediction: AP	80/82	9/158
Endoscopic prediction: non-AP	2/82	149/158

[1]AP= adenomatous polyps; non-AP= non-adenomatous polyps.

endoscopy, including 5 hyperplastic polyps, 2 inflammatory polyps and 2 lymphoid aggregates which were overdiagnosed and 2 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, high-resolution chromoendoscopy had a sensitivity of 97.5%, specificity of 94.3%, NPV of 98.69% and PPV of 89.9% (Table 4).

DISCUSSION

Given the incidence of colorectal cancer in the Western population and the recent thrust to implement broad-based screening methods, the issue of endoscopic resection of potentially cancerous or precancerous lesions assumes immense practical importance. It has been estimated that approximately two thirds of colorectal cancers arise from adenomatous polyps. The endoscopic detection and removal of polypoid tumors, therefore, have been emphasized to prevent the development of advanced colorectal cancers[2,15,16]. Since 10%-30 % of all colorectal polyps are non-neoplastic, it is very important to distinguish non-neoplastic from neoplastic lesions by colonoscopy, because removal or biopsy of non-neoplastic lesions would not only waste time and money but may also increase procedure-associated complications[6-8]. In particular, since reported data suggest that 80%-90% of diminutive colorectal polyps ($\leqslant$5 mm) are histologically hyperplastic, the ability to establish an immediate endoscopic diagnosis that is virtually consistent with the histology would be useful because it would obviate the need for biopsies during colonoscopy and also the need to remove all diminutive polyps, which is time consuming, expensive, and associated with risk.

Various data have been reported on the diagnostic abilities of conventional colonoscopy and magnified chromoendoscopy for such differentiation[11-15]. However, to the best of our knowledge, there is no prospective study on the accuracy for the differential diagnosis of non-neoplastic from neoplastic colorectal lesions using conventional colonoscopy and high-resolution chromoendoscopy consecutively.

In the present study, the diagnostic accuracy by conventional endoscopy and magnified chromoendoscopy using indigo carmine was 72.0% and 95.6%, respectively, with statistically significant difference ($P < 0.0001$). Fifty-seven of 240 lesions were misdiagnosed by conventional endoscopy, including 38 hyperplastic polyps, 8 inflammatory polyps and 4 lymphoid aggregates which were over-diagnosed and 7 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, conventional colonoscopy could show over 90% of the neoplastic lesions, but less than 70% of the non-neoplastic lesions, suggesting the possibility of excessive treatment, waste of time and resources (Table 3). Eleven of 240 lesions were misdiagnosed by magnified chromoendoscopy, including 5 hyperplastic polyps and 2 inflammatory polyps, 2 lymphoid aggregates which were over-diagnosed and 2 adenomas with mild atypia which were underdiagnosed. Magnified chromoendoscopy displayed 97.5% of the neoplastic lesions and 94.3% of the non-neoplastic lesions. Two (0.8%) false negative cases due to mixed polyps were diagnosed by magnified ehromoendoscopy.

Because the NPV was 98.6% indicating that some polyps were overlooked, it could be argued that high-resolution chromoendoscopy is not completely useful in clinical practice. Since no screening tests currently used have a NPV of 100%, the combination of magnified colonoscopy and dye spraying is the most reliable non-biopsy method for distinguishing the non-neoplastic from the neoplastic lesions at present. Moreover, with improved dyes and higher resolution chromoendoscope, better results might be obtainable. In this study all procedures were performed by a single operator who was well trained in magnified chromoendoscopy. The problem of a single operator study can represent the difficulty in deciding whether such results can be generalized. The time necessary for learning to recognize the mucosal crypt patterns is important, but has not been precisely reported. The endoscopist in this study had 5 years of experience in magnified endoscopy. In our previous experience, a foreign doctor without knowledge of pit patterns took only 3 months to acquire a differential diagnostic accuracy of over 90 %, which was similar to that of the well-trained endoscopists in this study (unpublished data). We believe that the time for learning could be shortened by showing trainees numerous typical endoscopic pictures of pit patterns before they perform the diagnostic procedure. However, this should be confirmed by future prospective studies including a large number of endoscopists.

Further questions include whether magnified endoscopy is as effective as total colonoscopy, how much time this adds to per case, and what is the cost associated with this procedure. In this study, with an instrument that was slightly more difficult to handle, intubation of the cecum was achieved for all patients without complications. Therefore, our results support the conclusion that magnified endoscopy is safe and can be used routinely. In our experience, the procedure usually only takes about 10-20 seconds for one polyp, and is less troublesome and time-wasting than colonoscopy.

In conclusion, the combination of colonoscopy and magnified chromoendoscopy is the most reliable nonbiopsy method for distinguishing non-neoplastic from neoplastic lesions. The use of this technique could decrease the time and resources required during colonoscopy by reducing the need for biopsy and resection of non-neoplastic polyps.

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RAPID COMMUNICATION

Phosphate-activated glutaminase activity is enhanced in brain, intestine and kidneys of rats following portacaval anastomosis

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Abstract

AIM: To assess whether portacaval anastomosis (PCA) in rats affects the protein expression and/or activity of glutaminase in kidneys, intestines and in three brain areas of cortex, basal ganglia and cerebellum and to explain the neurological alterations found in hepatic encephalopathy (HE).

METHODS: Sixteen male Wistar rats weighing 250-350 g were grouped into sham-operation control ($n = 8$) or portacaval shunt ($n = 8$). Twenty-eight days after the procedure, the animals were sacrificed. The duodenum, kidney and brain were removed, homogenised and mitochondria were isolated. Ammonia was measured in brain and blood. Phosphate-activated glutaminase (PAG) activity was determined by measuring ammonia production following incubation for one hour at 37℃ with O-phthalaldehyde (OPA) and specific activity expressed in units per gram of protein (μkat/g of protein). Protein expression was measured by immunoblotting.

RESULTS: Duodenal and kidney PAG activities together with protein content were significantly higher in PCA group than in control or sham-operated rats (duodenum PAG activity was 976.95±268.87 μkat/g of protein in PCA rats *vs* 429.19±126.92 μkat/g of protein in sham-operated rats; kidneys PAG activity was 1259.18 ± 228.79 μkat/g protein in PCA rats *vs* 669.67± 400.8 μkat/g of protein in controls, $P < 0.05$; duodenal protein content: 173% in PCA *vs* sham-operated rats; in kidneys the content of protein was 152% in PCA *vs* sham-operated rats). PAG activity and protein expression in PCA rats were higher in cortex and basal ganglia than those in sham-operated rats (cortex: 6646.6 ± 1870.4 μkat/g of protein *vs* 3573.8 ± 2037.4 μkat/g of protein in control rats, $P < 0.01$; basal ganglia, PAG activity was 3657.3 ± 1469.6 μkat/g of protein in PCA rats *vs* 2271.2 ± 384 μkat/g of protein in sham operated rats, $P < 0.05$; In the cerebellum, the PAG activity was 2471.6 ± 701.4 μkat/g of protein *vs* 1452.9 ± 567.8 μkat/g of protein in the PCA and sham rats, respectively, $P < 0.05$; content of protein: cerebral cortex: 162% ± 40% *vs* 100% ± 26%, $P < 0.009$; and basal ganglia: 140% ± 39% *vs* 100% ± 14%, $P < 0.05$; but not in cerebellum: 100% ± 25% *vs* 100% ± 16%, P = ns).

CONCLUSION: Increased PAG activity in kidney and duodenum could contribute significantly to the hyperammonaemia in PCA rats, animal model of encephalopathy. PAG is increased in non-synaptic mitochondria from the cortex and basal ganglia and could be implicated in the pathogenesis of hepatic encephalopathy. Therefore, PAG could be a possible target for the treatment of HE or liver dysfunction.

Key words: Hyperammonaemia; Minimal hepatic encephalopathy; Glutamine; Protein content; Sham-operated

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INTRODUCTION

Hyperammonaemia plays a major role in the pathogenesis of hepatic encephalopathy (HE). Phosphate-activated glutaminase (PAG) catalyzes the hydrolysis of glutamine (Gln) to yield glutamate (Glu), energy, nucleotide synthesis and ammonia. Two main isoforms of PAG have been described: the kidney-type (K-PAG) and the liver type (L-PAG). The K-PAG has been found in kidney, brain and villi enterocytes, while L-PAG is restricted to the liver. Hyperammonaemia is largely considered to be derived from urea breakdown by intestinal bacteria, thus most treatments have been focussed on reducing ammonia production from colon bacteria[1]. However, some data suggest that small intestine plays an important role in ammonia production during the pathogenesis of hepatic encephalopathy[2]. Indeed, duodenal PAG activity has been found to be nearly four times higher in cirrhotic patients than in healthy controls, and moreover it is closely related to minimal hepatic encephalopathy[3]. In rats, the highest PAG activity along the length of gastrointestinal tract has been measured in the small intestine[4]. Lower but still substantial activity (15% of total PAG activity) has been found in large intestine. PAG activity distribution along the gastrointestinal tract in humans is similar to that in rats[5]. PAG plays another important role at renal level. Glutamine is filtered and reabsorbed in the proximal convoluted tubule where it is deamidated by PAG. Two thirds of this ammonia production is excreted in urine. This process is pH sensitive and helps to maintain acid-base homeostasis and to excrete nitrogen[6]. However, PAG activity in kidney remains largely unexplored in portacaval shunted rats. Distribution and location of PAG in brain as well as its role in the pathogenesis of HE, are widely controversial, while PAG activity in portacaval shunted rats still remains unknown. Portacaval anastomosis (PCA; or portacaval shunt PCS) in rats is widely accepted as a model of type B hepatic encephalopathy that mimics minimal hepatic encephalopathy in humans. The aim of this study was to assess whether portacaval anastomosis in rats affects the expression and/or activity of glutaminase in kidney, intestines and the astrocytes of three brain areas (cortex, basal ganglia and cerebellum) involved in the neurological alterations of hepatic encephalopathy.

MATERIALS AND METHODS

Animal surgery

Sixteen male Wistar rats (n = 16) weighing 250-350 g were randomly allocated into sham operation controls (n = 8) or portacaval shunt (n = 8). All animals were pair fed two weeks before and four weeks after operation and kept under standard laboratory conditions. Both PCA and sham-operated rats were fasted overnight in single wire-net floor cages with free access to tap water. All animal procedures were approved by our institution and met the guidelines of Spain (RD 223 erased 14th March 1998) and European (Directive 86/609/CEE) Union for care and management of experimental animals.

Rats were operated under general anaesthesia with isofluorane to avoid liver metabolism of intravenous agents and end-to-side portacaval shunt was performed as previously described[7]. Briefly, after middle laparotomy for viscera exteriorization, inferior vena cava and portal veins were exposed, dissected and clamped laterally together with a Satinsky clamp. Longitudinal incisions in both veins and lateral anastomosis with running suture as usual were performed. Finally, portal trunk was tied and cut next of liver hilum, turning the portacaval lateral shunt in functionally terminal. Sham-operation was performed following laparotomy, the inferior vena cava was isolated and clamped for 30 s. Sham-operated animals served as controls.

Determination of ammonia in brain and blood

Ammonia was measured in cerebral cortex and blood as previously described[8]. Cerebral cortex was homogenized and deproteinised in 5 volumes of ice-cold 100 g/L trichloroacetic acid, and kept on ice for 15 min. After centrifugation at 12 000 r/min for 10 min at 4 ℃, the supernatants were collected, neutralized with 2 mol/L $KHCO_3$ and centrifuged at 12 000 r/min for 10 min at 4 ℃. The neutralized supernatants were used to measure ammonia in μmole/g tissue. Blood (150 μL) was taken from the tail vein the third week after surgery. Blood samples were deproteinized with one volume of ice-cold 100 g/L trichloroacetic acid and kept on ice for 15 min. After centrifugation at 12 000 r/min for 10 min at 4 ℃, the supernatants were collected, neutralized with 2 mol/L $KHCO_3$ and centrifuged at 12 000r/min for 10 min at 4 ℃. The neutralized supernatants were used to measure ammonia. In a final volume of 100 μL, the reaction mixture contained 50 μL or 60 μL of sample, 30 mmol/L α-ketoglutarate, 0.5 mmol/L nicotinamide adenine dinucleotide (reduced form) in potassium phosphate buffer (pH 8.0). After recording of the initial fluorescence, reactions were started by the addition of 5 μg of glutamate dehydrogenase (Boehringer Mannheim, Germany) and monitored by the fluorimeter (Fluoroskan Ascent; Labsystems; Oy, Helsinki, Finland) for at least 70 min. Standards containing up to 25 nmol of ammonia were included in each assay. Assays were performed in Costar 96-well UV plates (cat. No. 3635; Corning Costar Corporation, Cambridge, MA).

Animal handling and brain tissue preparation

All procedures were carried out in the cold room at 2℃-4℃. The rats were killed by cervical dislocation. The skull of each rat was opened to remove blood from the surface of the tissue and this procedure needed to be performed in <30 s. The tissue was placed in 5 mL of isolation medium in a Petri dish maintained in an ice bath. The brain was chopped with fine scissors and the chopped material was washed frequently with isolation medium to remove blood. Meanwhile, the first portion of small intestine (3 cm of length) was removed and placed in 5 mL of isolation medium in Petri dish maintained in an ice bath. The duodenum was washed and mucosa was removed by glass film and quickly frozen in liquid air. Samples of kidney were similarly obtained. Tissue samples were then homogenized manually in 1 mL of isolation medium per 100-150 mg of tissue using a Dounce

homogenizer fitted with a Teflon pestle having a total clearance of 0.1 mm. Usually, six up- and down-strokes were sufficient to generate a rough homogenate which was then diluted with isolation medium to a final volume of 1.2 mL and homogenized further with four up- and down-strokes.

Preparation of mitochondria

The procedure for isolation of brain mitochondria was based on previously reported methods[9] except for 1 mmol/L ethylene glycol-bis (β-aminoethyl ether) tetra-acetic acid (EGTA) being used in the homogenisation medium instead of EDTA. The homogenate was centrifuged at 2 000 r/min for 3 min, the pellet was washed with 400 μL of homogenisation medium and re-centrifuged at 2 000 r/min for 3 min. Both supernatants were pooled and centrifuged for 8 min at 12 000 r/min to obtain the crude mitochondrial pellet. The pellet was suspended in 300 μL of the 30 g/L Ficoll medium (see below) and layered onto 1.2 mL of 60 g/L Ficoll medium and centrifuged at 12 000 r/min for 30 min. The 60 g/L Ficoll medium contained 6% (w/w) Ficoll, 0.24 mol/L mannitol, 0.06 mol/L sucrose, 0.05 mmol/L K-EDTA and 10 mmol/L Tris-HCl, pH 7.4. The 30 g/L Ficoll medium was the 60 g/L Ficoll medium diluted 1:1 with glass re-distilled water. The loose, fluffy, white upper layer of the pellet was removed, the remaining brown pellet was re-suspended in isolation medium without EGTA and the suspension was centrifuged at 12 000 r/min for 10 min. The pellet was re-suspended in incubation medium (isolation medium with 2.5 mL/L buffer containing protease inhibitor, 7 g/L Triton X-100, 5 mmol/L β-mercapto-ethanol) to obtain a protein concentration in the range of 5-10 g/L. After incubation on a mixing wheel for 30 min at 4 ℃, the samples were frozen at -80 ℃ for batched activity measurement.

PAG activity assay

Mitochondrial protein was measured by the method of Bradford *et al*[10] with bovine serum albumin (BSA) as standard. Briefly, 25 μL of mitochondrial solution was added to 35 μL of reaction medium (150 mmol/L K_2HPO_4, pH 8; 171 mmol/L L- GLn; 1 mmol/L NH_4Cl; pH 8). After incubation for 60 min the reaction was stopped with 10 μL of 100 g/L trichloroacetic acid (TCA). Blanks were prepared separately following the incubation of the reaction medium and samples were mixed before the addition of TCA. When the sample-mixture reaction was stopped, the reaction mixture was placed in ice for 15 min and then centrifuged at 12 000 r/min for 5 min at 4 ℃. The micro-titre plate was loaded with 5 μL of supernatant and 150 μL of OPA reagent (0.2 mol/L K_2HPO_4, pH 7.4; 56 mL/L ethanol; 10 mmol/L *O*-phthaldialdehyde; 0.4 mmol/L *β*-mercapto-ethanol). The plate was incubated in dark at room temperature for 45 min. Standards of NH_4Cl were prepared to concentrations of 50-300 mg/L. Absorbance was measured at 405 nm with a spectrophotometer (R&D System, Palo Alto, USA). Specific activities of enzymes were expressed in international units per gram (μkat/mg) of mitochondrial homogenate protein.

Analysis of glutaminase protein content by immunoblotting

Samples from sham-operated rats or rats with PCS were homogenized in medium containing 66 mmol/L Tris-HCl (pH 7.4), 10 g/L SDS, 1 mmol/L EGTA, 100 ml/L glycerol, 1 mmol/L sodium orthovanadate and 1 mmol/L sodium fluoride and the protein concentration was determined by the bicinchonic acid method (Pierce, Rockford, IL, USA). Samples were subjected to gel electrophoresis and immunoblotting as previously described[11] using isoform-specific polyclonal antibodies raised in rabbits against K- glutaminase proteins diluted at 1:1000. After incubation with anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, Germany) and development with alkaline phosphatase colour developer (Sigma, Germany), the image was captured using the Gel Printer Plus System (TDI, Madrid, Spain) and the densities of the spots were measured using the Intelligent Quantifier ™ software Version 2.5.0 (BioImage®, Madrid, Spain). Results were relativized to the optical density respect to controls.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using the SPSS 11.0 software (spss, Chicago, IL). Differences in glutaminase activity or protein content were analysed by Student *t*-test. $P \leqslant 0.05$ was considered statistically significant for all tests applied.

RESULTS

Ammonia measurement

Ammonia was significantly higher in PCA group than in control rats. Plasma ammonia level was 166 ± 51 μmol/L in PCA rats and 83 ± 12 μmol/L in control rats ($P < 0.05$). In the cortex, brain ammonia was 0.9±0.4 μmol/g of tissue in PCA rats and 0.3 ± 0.1 μmol/g of tissue in sham operated rats ($P < 0.05$).

PAG activity in duodenum and kidneys

Duodenal and kidney PAG activities were significantly higher in PCA group than in control rats. In the duodenum, PAG activity was 976.95 ± 268.87 μkat/g of protein in PCA rats and 429.19 ± 126.92 μkat/g of protein in sham-operated rats ($P < 0.05$). In mitochondria from kidneys, PAG activity was 1259.18 ± 228.79 μkat/g protein in PCA rats and 669.67 ± 400.8 μkat/g of protein in controls ($P < 0.05$).

Glutaminase protein content in intestine and kidneys

There was a significant effect of PCA on PAG protein in duodenum. The content of protein was 173% in PCA compared to sham-operated rats and in kidneys the content of protein was 152% in PCA compared to sham-operated rats (Figure 1).

PAG activity in brain

The PAG activity was 6646.6 ± 1870.4 μkat/g of protein in the cortex of PCA rats and 3573.8 ± 2037.4 μkat/g of protein in that of control rats ($P < 0.01$). The PAG activity was 3657.3 ± 1469.6 μkat/g of protein in basal ganglia

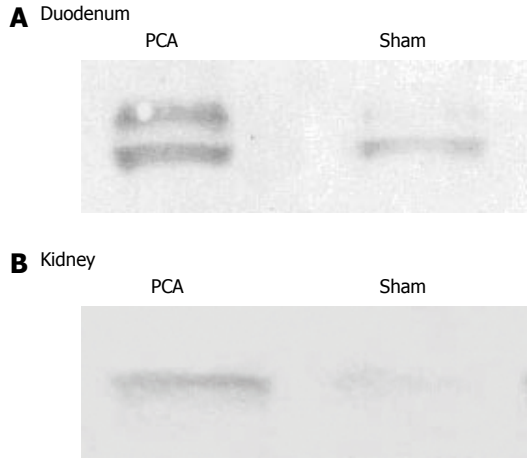

Figure 1 Representative immunoblotting from duodenum (**A**) and kidneys (**B**). Glutaminase content in enterocytes from rats with portacaval shunt (PCA) and 6 control rats (sham) homogenised and subjected to immunoblotting, 10 μg of protein applied in each lane.

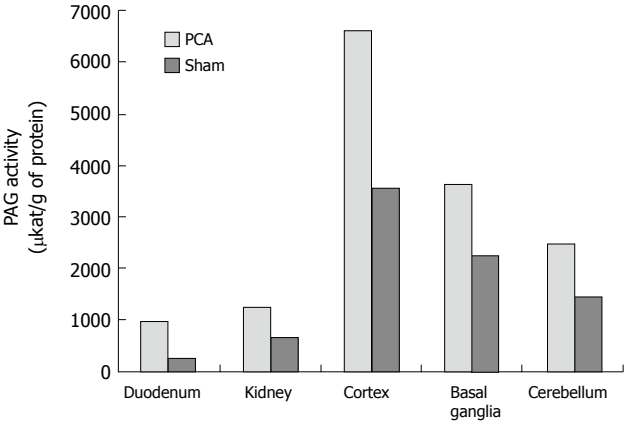

Figure 2 PAG activity (kat/g of protein) in duodenum, kidneys and brain of PCA and control rats (sham).

of PCA rats and 2271.2 ± 384 μkat/g of protein in that of sham-operated rats ($P < 0.05$). In the cerebellum, the PAG activity was 2471.6 ± 701.4 μkat/g of protein and 1452.9 ± 567.8 μkat/g of protein in the PCA and sham-operated rats, respectively ($P < 0.05$). In PCA rats, the PAG activity was increased up to 186% in cortex, 161% in basal ganglia and 170% in cerebellum compared to sham-operated rats. The highest activity was found in the cortex (Figure 2).

Glutaminase protein content in cerebral cortex and basal ganglia

The content of glutaminase was significantly increased in cerebral cortex of PCA rats compared to sham-operated animals (162% ± 40% and 100% ± 26%; $P < 0.009$) and the content of glutaminase was significantly higher in basal ganglia of PCA rats than in that of sham-operated animals (140% ± 39% and 100%±14%; $P < 0.024$), but there was no significant difference between the groups after PCA in cerebellum (100% ± 25% and 100% ± 16%; P = NS) (Figure 3).

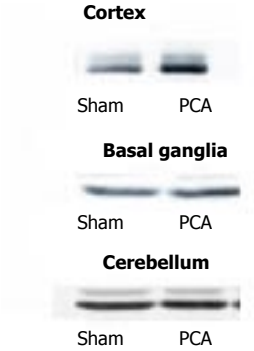

Figure 3 Glutaminase content in cerebral cortex and basal ganglia. Cerebral cortex basal ganglia or cerebellum from 7 rats with portacaval shunt (P) and 6 control rats (C) was homogenised and subjected to immunoblotting, 10 μg of protein was applied in each lane. The codes under the bands of representative blots indicate the different rats with portacaval shunt (P) or control rats (C).

DISCUSSION

The PCA performed in rats is widely accepted as a model of liver dysfunction. In this study, PAG activity was increased in kidney and duodenum, which could contribute significantly to systemic hyperammonaemia. PAG activity was increased in cortex and basal ganglia, which might be responsible for brain hyperammonemia and intra-mitochondrial ammonia levels derived from glutamine hydrolysis that are maintained because successful detoxification by glutamine-synthetase is precluded.

During the 2nd half of the 20th century, hyperammonaemia was considered to be derived from urea breakdown by intestinal bacteria and the majority of treatments are targeted against bacteria-derived ammonia from the colon[1]. However, the hypothesis was not universally accepted[12]. Hyperammonaemia following portacaval shunting in rats has been found to be similar in germ-free as well as in non-germ-free animals [13, 14], providing support to the concept that hyperammonaemia and encephalopathy could develop without participation of bacteria[15]. The highest hyperammonaemia has been found in portal-drained viscera and derived mainly from glutamine deamidation[16]. Hence, increased PAG activity in small intestine could explain these observations at least in part. Moreover, increased PAG in duodenum has been demonstrated in cirrhotic patients suffering from minimal hepatic encephalopathy[3].

Recently, renal ammoniagenesis has been implicated in several forms of hepatic encephalopathy. In patients suffering from hepatic encephalopathy due to variceal bleeding or overdose of diuretics, ammonia production from kidney seems to be the main factor involved in the development of hepatic encephalopathy[17]. Glutamine is filtered in the glomeruli and enters the lumen of the nephron. The filtered glutamine is reabsorbed in the proximal tubule where glutamine is deamidated by PAG. The main factor in the regulation of kidney PAG activity seems to be pH. During metabolic acidosis, PAG activity increases which induces higher excretion of ammonia[18]. In hepatic encephalopathy, metabolic alkalosis is observed more frequently than acidosis and increased PAG activity in kidney could be a protective mechanism. This PAG increase could be even more protective than the event associated with the systemic hyperammonaemia. However, this possibility needs to be explored in greater detail in future studies.

Ammonia reaching the brain can be detoxified to

glutamine only in astrocytes due to the predominance of glutamine synthetase in these cells. Glutamine accumulation as a by-product of ammonia metabolism has been implicated in the pathogenesis of hepatic encephalopathy[19]. Glutamine is an osmotic amino acid and plays a major role in the regulation of cell volume. A raised peak of glutamine-glutamate/creatine ratio is a typical feature of brain spectroscopic magnetic resonance imaging in hepatic encephalopathy. However, glutamine synthetase has not been found to be increased in brain of portacaval shunted rats[20]. Indeed, administration of amino acid mixtures induces hyperammonaemia, raises glutamine peak in the brain and is associated with impairment in neuropsychological function[21]. Also, use of methionine-sulfoxamine blocks the activity of glutamine synthetase and improves abnormalities induced by ammonia, such as seizures or astrocytes swelling[22]. Nevertheless, accumulation of glutamine following treatment with drugs that are able to block N-methyl-D-aspartate (NMDA)-receptors has not been shown to be associated with neurological impairment[23]. Indeed, in astrocyte culture, glutamine metabolism is linked to free radical production and oxidative stress and this may represent a key mechanism in ammonia neurotoxicity [24]. Thus, glutamine accumulation in the brain is neither a pathological event *per se* nor a safe ammonia detoxification pathway. Glutamine accumulating in the astrocytes can be considered as a "Trojan horse" leading circuitously to neurological impairment. In cultured astrocytes, glutaminase inhibitors such as 6-diazo-5-oxo-L-norleucine (DON) induce a complete blockade of glutaminase activity and preempt free radical production and neurotoxicity induced by ammonia[25]. Most of the glutamine in astrocytes is metabolized by mitochondrial PAG[26]. Since ammonia induces free radical production, the PAG can be implicated in free radical production. The inhibition of this enzyme could be a new therapeutic target. However, DON has also been reported to inhibit γ-glutamyl-transpeptidase, increase glutamine release, inhibit transport of glutamine into cells and block the transport of glutamine into mitochondria. All these mechanisms could induce a decrease in the amount of glutamine available for hydrolysis[27]. The distribution of K-type PAG in the brain has been strongly debated. Some studies have reported that PAG is absent in astrocytes of cerebellum[28]. In the current study, PAG activity in non-synaptic mitochondria from the cerebellum showed the lowest activity in the brain and no differences were observed between PCA and sham-operated rats using immunoblotting. Thus, PAG could be detected in cerebellum, but at a low-level in comparison to other areas such as basal ganglia or cortex.

In summary, PAG is enhanced in the intestine and kidney of PCA rats and induces hyperammonaemia and hepatic encephalopathy. Furthermore, PAG activity and glutaminase content are increased in astrocytes from cortex and basal ganglia. Mitochondrial glutaminase activity in astrocytes could be implicated in the production of ammonia. The induction of the mitochondrial permeability transition and free radicals production as the end product of glutamine metabolism could be responsible at least in part for the pathogenic effect observed in hepatic encephalopathy. Hence, PAG might be a new therapeutic target in the management of hepatic encephalopathy. Further studies using PAG inhibitors or PAG knock-out mice help clarify the role of increased PAG expression in the pathophysiology of hepatic encephalopathy.

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RAPID COMMUNICATION

Lamivudine therapy for children with chronic hepatitis B

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Abstract

AIM: To assess the effectiveness and side-effects of lamivudine therapy for children with chronic hepatitis B (CHB) who fail to respond to or have contraindications to interferon-α (IFN-α) therapy.

METHODS: Fifty-nine children with CHB were treated with 100 mg lamivudine tablets given orally once daily for 12 mo. Alanine aminotransferase (ALT) activity was evaluated monthly during the therapy and every 3 months after its discontinuation. HBe antigen, anti-HBe antibodies, HBV DNA level in serum were evaluated at baseline and every six months during and after the lamivudine therapy. Sustained viral response (SVR) to lamivudine therapy was defined as permanent (not shorter than 6 mo after the end of the therapy), namely ALT activity normalization, seroconversion of HBeAg to anti-HBe antibodies, and undetectable viral HBV-DNA in serum (lower than 200 copies per mL). The analysis of the side-effects of the lamivudine treatment was based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and laboratory tests performed during clinical visits monthly during the therapy, and every 3 mo after the therapy.

RESULTS: ALT normalisation occurred in 47 (79.7%) patients between the first and 11th mo of treatment (mean 4.4 ± 2.95 mo, median 4.0 mo), and in 18 (30.5%) of them after 2 mo of the therapy. There was no correlation between the time of ALT normalization and the children's age, the age of HBV infection, the duration of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, serum HBV DNA level, and lamivudnie dose per kg of body weight. HBeAg/anti HBe seroconversion was achieved in 27.1% of cases. The higher rate of seroconversion was connected with lower serum HBV DNA level and longer duration of HBV infection. There was no connection between HBeAg/anti HBeAb seroconversion and the children's age, age of HBV infection, grading, staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. No complaints or clinical symptoms were observed during lamivudine therapy. Impairment of renal function or myelotoxic effect was noted in none of the patients.

CONCLUSION: One year lamivudine therapy for children with chronic hepatitis B is effective and well tolerated. Seroconversion of HBeAg/HBeAb and SVR are connected with lower pre-treatment serum HBV DNA level.

Key words: Chronic hepatitis B; Children; Lamivudine

Liberek A, Szaflarska-Popławska A, Korzon M, Łuczak G, Góra-Gębka M, Łoś-Rycharska E, Bako W, Czerwionka-Szaflarska M. Lamivudine therapy for children with chronic hepatitis B. *World J Gastroenterol* 2006; 12(15): 2412-2416

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INTRODUCTION

Hepatitis B virus (HBV) infection is still an important problem due to its high incidence and may lead to chronic hepatitis[1]. Up to 90% of infected children develop chronic hepatitis[2]. Chronic hepatitis B greatly increases the risk of liver cirrhosis or hepatocellular carcinoma[3]. Spontaneous seroconversion of HBeAg/HBeAb during the course of chronic hepatitis B is observed only in less than 10% of children, and total recovery with the elimination of all virus antigens and the presence of anti-HBs occurs in approximately 2% of cases[4]. In recent years lamivudine treatment for chronic hepatitis B has been recommended for patients who fail to respond to IFN-α therapy or have contraindications for this therapy[5]. However clinical data concerning nucleotide analogue treatment for CHB children are lacking.

The aim of the present study was to analyze prospectively the results, tolerance, and side-effects of lamivudine therapy for children with chronic hepatitis B who fail to respond to or have contraindications for IFN-α treatment.

Table 1 Characteristics of the patients

	n (%)	Mean	Median
Sex			
Boys	48 (81.4)		
Girls	11 (18.6)		
Age (yr)	6-18	10.5±3.24	10
Age of HBV infection (yr)	1-14	3.7±3.09	3
Duration of HBV infection (yr)	1-16	6.8±3.09	6
Completed previous IFN-α treatment			
Yes	51 (86.4)		
No	8 (13.6)		
ALT activity before treatment (IU/L)	20-664	101±96.3	76
Inflammation activity score			
Grade 1	24 (40.7)		
Grade 2	33 (55.9)		
Grade 3	2 (3.4)		
Grade 4	0		
Staging			
Stage 0	7 (11.9)		
Stage 1	37 (62.7)		
Stage 2	12 (20.3)		
Stage 3	2 (3.4)		
Stage 4	1 (1.7)		
Serum HBV DNA level (copies/mL)	200-200 000	135 632±81 018	200 000
Lamivudnie dose (mg per kg of body weight)	1.3-4.1	3.0±0.85	2.9

MATERIALS AND METHODS

Fifty-nine children, 48 boys and 11 girls, aged 6 - 18.0 years were included in the study. The age of HBV infection varied from 1 to 14 years and the known duration of infection was 1-16 years (Table 1). The precise route of infection was not determined. Most of the children had a history of multiple hospital admissions. None of these children suffered from onco-hematological disorders. Fifty-three children were previously treated with recombinant (3 million units) IFN-α given subcutaneously, three times a week for 20 wk, recommended by the Polish Working Liver Group[6]. Fifty-one of them completed the therapy, in 2 cases the therapy was discontinued at the 8th and 12th wk due to repeated seizures. Interferon therapy was completed 1 - 7.5 years before the present study (mean 4.0 ± 1.8 years, median 4.0 years). Six patients (10.2%) were not previously treated because of relative contraindications for IFN-α therapy, including high grade fibrosis on liver biopsy in 3 cases and epilepsy in 1 case.

The inclusion criteria for lamivudine treatment were increased aminotransferase activities in serum noted at least three times during the last six months before therapy, the presence of HBsAg and HBeAg in the blood, measurable (above 200 genome copies/mL) HBV-DNA in serum for at least six months prior to the study, negative HCV-RNA and anti-HCV antibodies in serum, and evidence of inflammation on liver biopsy performed within 24 mo before the study.

Patients with coexisting clinically significant illness or other types of liver disease, or having received antiviral agents, immunomodulatory drugs within the previous 6 mo were excluded from the study. The teenage pregnant girls were excluded and advised not to get pregnant during and after 6 mo after the lamivudine therapy.

ALT activity as well as total and direct bilirubin, alkaline phosphatase (ALP) and gammaglutamyl-transpeptidase (GGT) activity in serum were measured before treatment, monthly during and every 3 mo after the therapy by routine laboratory method. Serological markers of HBV infection were analyzed before and every six months during and after the therapy by immunoenzymatical methods: HBeAg, anti-HBe and anti-HBs by Roche's diagnostics tests while HBsAg by Micro Elisa tests. HBV-DNA serum concentration was measured in all patients before and every six months during and after the lamivudine therapy by quantitative PCR method using the Roche Cobas Amplicor HBV Monitor Assay (Roche Diagnostics, Pleasanton, USA). Activity of inflammation (grading) and fibrosis (staging) of liver biopsy was classified according to Scheuer's scale modified by International Working Party in 1995[7].

All patients were treated with 100 mg lamivudine tablets given orally once daily for 12 mo (Zeffix, GlaxoSmithKline Pharmaceuticals, SA). A single dose of lamivudine varied from 1.3 mg/kg to 4.1 mg/kg (Table 1)

The analysis of the side-effects of the lamivudine treatment was based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and laboratory tests (level of hemoglobin, blood cell count, urea and creatinine level) performed monthly during and every 3 mo after the therapy. The duration of clinical observation was at least 6 mo after the therapy in all cases.

Sustained viral response (SVR) to lamivudine therapy was defined as permanent (not shorter than 6 mo after the end of the therapy): ALT activity normalization, seroconversion of HBeAg to anti-HBe antibodies, and undetectable viral HBV-DNA in serum (lower than 200 copies per mL).

In patients with ALT elevation during the lamivudine therapy mutations in the YMDD (tyrosine, methionine, aspartate, and asparate) motif of the reverse-transcriptase domain in the HBV polymerase gene were assessed by polymerase chain reaction and restriction-fragment-length polymorphism assay. Analysis of HBV genotype was performed only in these patients because of economical reasons.

The results of the study were statistically analyzed using Statistica 5.77 (StatSoft, Inc., Tulsa, OK, USA). The differences in frequency were analyzed using χ^2 test with Yate's correction if necessary. The differences between groups were achieved using U Mann-Whitney's test. $P < 0.05$ was considered statistically significant.

Children over 12 years of age and their parents or legal guardians provided their written informed consent. The study was approved by the Ethics Committee of the Medical University of Gdańsk.

Table 2 Statistical analysis of factors predicting response to lamivudine treatment

Factors that may predict response	ALT normalisation	HBe/anti-HBe seroconversion	Sustained viral response
Sex	$\chi^2_{(Y)}$=0.37, P=0.54	$\chi^2_{(Y)}$=0.13, P=0.72	$\chi^2_{(Y)}$=0.01, P=0.76
Previous IFN-α treatment	$\chi^2_{(Y)}$=0.70, P=0.76.	$\chi^2_{(Y)}$=3.29, P=0.07	$\chi^2_{(Y)}$=4.42, P=0.04
Children's age	Z=0.10, P=0.92	Z=1.83, P=0.07	Z=2.28, P=0.02
Age of HBV infection	Z=0.70, P=0.49	Z=-1.49, P=0.14	Z=-1.40, P=0.15
Duration of HBV infection	Z=0.13, P=0.89	Z=2.99, P=0.002	Z=3.33, P<0.001
Inflammation activity score (grading)	Z=0.23, P=0.82	Z=1.56, P=0.12	Z=1.76, P=0.08
Staging	Z=0.88, P=0.38	Z=1.46, P=0.14	Z=1.69, P=0.09
ALT activity before treatment	Z=-0.48, P=0.63	Z=1.49, P=0.14	Z=1.23, P=0.23
Serum HBV DNA level	Z=-0.80, P=0.07	Z=-3.29, P=0.001	Z=-3.22, P=0.001
Lamivudnie dose per kg of body weight	Z=-0.46, P=0.64	Z=-1.75, P=0.08	Z=-1.59, P=0.11

RESULTS

ALT activity before the lamivudine therapy varied from 20 to 664 IU/L (Table 1). The ALT level was lower than 100 U/L in 42 (71.2%) patients and higher than 100 U/L in 17 (28.8%) patients. All 59 children who completed the 12-mo therapy had normal serum, total and direct bilirubin, and ALP. GGT level in serum was slightly elevated only in 3 (5.1%) patients.

Inflammation activity in liver biopsy specimens was found at either low or medium levels in 96.6% of patients: grade 1 in 24 and grade 2 in 33 children. Only in 2 patients grade 3 inflammation activity was observed (Table 1). No liver fibrosis was found in 7 patients. Fibrosis of stages 1-4 was found in 37, 12, 2, and 1 patient, respectively (Table 1).

Before lamivudine treatment HBV-DNA serum level ranged between 200-200 000 copies/mL. HBV-DNA level was over 200 000 copies/mL in 33 children (55.9%), 10 000-200 000 copies/mL in 16 (27.1%) children, and below 10 000 copies/mL in 10 (16.9%) children (Table 1).

ALT normalization was achieved in 47 (79.7%) patients at the end of therapy. It occurred mostly between the first and the 11th mo of treatment (mean 4.4 ± 2.95 mo, median 4.0 mo). ALT normalization was observed in 37 of 48 boys and 10 of 11 girls, in 43 of 53 children previously treated and 4 of 6 not treated with IFN-α. There was no connection between the rate of ALT normalization and the children's age, age of HBV infection, duration of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, serum HBV DNA level, and lamivudnie dose per kg of body weight. Statistical results are shown in Table 2.

HBeAg/anti-HBeAb seroconversion was achieved in 16 cases (27.1%) at the end of therapy. It occurred mostly after 12 mo of treatment. This seroconversion was observed in 13 of 48 boys and 3 of 11 girls, in 12 of 53 children previously treated and 4 of 6 not treated with IFN-α. The higher rate of seroconversion was connected with longer duration of HBV infection (median 9 *vs* 5 years) and lower serum HBV DNA level (median 50 000 *vs* 200 000 copies/mL). There was no connection between HBeAg/anti-HBeAb seroconversion and the children's age, age of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. Statistical results are shown in Table 2. HBsAg/anti-HBsAb seroconversion was observed six months after the end of the therapy only in one child (1.7%). In 14 patients (23.7%) with ALT normalization and HBeAg/anti-HBeAb seroconversion, sustained viral response (SVR) was achieved at the end of therapy. In these cases HBV DNA level in serum was lower than 200 copies/mL. In two patients with ALT normalization and HBeAg/anti-HBe seroconversion, the serum HBV DNA level remained high (14 400 and 145 000 copies/mL). SVR was observed in 11 of 48 boys and 3 of 11 girls, and more frequently achieved in children previously treated with IFN-α. The rate of SVR was connected with older children's age (median 12 *vs* 9 years), longer duration of HBV infection (median 9 *vs* 5 years), and lower serum HBV DNA level (median 50 000 *vs* 200 000 copies/mL). There was no connection between SVR and the age of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. Statistical results are also shown in Table 2.

No complaints or clinical symptoms were observed during the lamivudine therapy. Slight and transient increase of ALT activity was observed in 4 children (6.8%) between the 3rd and the 12th mo of treatment. No association with hyperbilirubinemia or other signs of hepatic decompensation was found in all cases. Mutations in the YMDD were detected in 2 of 4 patients with ALT elevation during the lamivudine therapy.

Lamivudine did not show myelotoxic effect in treated children. There were no significant differences between erythrocyte or leukocyte peripheral blood count, platelet count, and hemoglobin level during or after the therapy.

Impairment of renal function was observed in none of the patients.

DISCUSSION

This study presented an analysis of the outcome, tolerance and side-effects of lamivudine therapy for children with chronic hepatitis B, who failed to respond to or had contraindications for IFN-α treatment. Up till now IFN-α is the therapy of first choice for children with chronic hepatitis B in Poland. However the treatment with IFN-α is uncomfortable (especially in children) and has many different side effects[8].

Lamivudine is the first oral antiviral therapy for chronic hepatitis B. Positive results of this treatment in adult patients have made lamivudine therapy possible in children with chronic hepatitis B[9-11].

The results of international research conducted in children with chronic hepatitis B have proved that a 52-wk course of lamivudine therapy results in the significantly higher rate of viral response. Furthermore, SVR with HBs Ag/HBsAb seroconversion and ALT normalization has also been observed[12]. There are also some other data confirming positive biochemical and viral response in children with chronic hepatitis B treated with lamivudine[13,14].

This study demonstrated that 100 mg lamivudine treatment for 12 mo resulted in a 23.7% sustained virologic response. Special stress must be put on the fact that 86% of patients did not respond to IFN-α therapy.

Lamivudine therapy is mainly used for children with chronic hepatitis who fail to respond to IFN-α[11-15].

Other nucleoside analogues (like adefovir) used in the treatment of adult patients with chronic hepatitis, are not widely accessible for pediatric patients[16,17]. Data on the results of combined IFN-α and lamivudine therapy vary[18-21], seem no more effective than monotherapy with either IFN-α or lamivudine.

Lamivudine has been proved to be more effective than IFN-α for chronic hepatitis HBeAg-minus[22,23]. Most trials on the effectiveness of lamivudine therapy in both adults and children with chronic hepatitis B showed that ALT normalization is significantly more frequent even though it is often not associated with the viral response[10-16].

In the present group of children, ALT normalization within the first 11 mo of therapy was observed in almost 80% of patients and in 30% of children in the first 2 mo. No connection was noted between the time of ALT normalization and clinical data, biochemical tests, histopathological changes in the liver tissue, viral load, or lamivudine dose per kg body weight. Jonas *et al*[12] showed that the median time of ALT normalization was 24 wk.

In our group of patients, HBeAg/HBeAb seroconversion was observed in 27.1% of children and after 12 mo of treatment in most cases. Only in one patient, HBsAg/HBsAb seroconversion took place 6 mo after the lamivudine therapy. The low rate of positive response to lamivudine therapy expressed by HBsAg/HBsAb seroconversion is consistent with other reports[12,13,15]. SVR was noted in 23.7% of patients. ALT normalization and HBeAg/HBeAb seroconversion were still accompanied with high viral load. The results of lamivudine therapy are similar to other reports[12,24]. Some authors investigating lamivudine therapy effectiveness demonstrated a higher viral response rate of 36-44%[13,14]. However, Kocak *et al*[11] observed HBeAg/HBeAb seroconversion only in 5% of cases while viral load significantly decreased in 90% of cases[11].

In the analyzed group of patients the positive response to the lamivudine treatment was connected with the older age of patients and lower serum DNA level before therapy. The similar connection between SVR and the pretreatment viral load has also been observed by other authors[12,13]. On the contrary to the published data[9,25], no connection between SVR and pretreatment ALT activity or grading was noted in our study.

Special attention must be paid to the histopathological improvement within the liver tissue and in ALT activity in patients with no viral response after lamivudine therapy[9].

Liver biopsy after lamivudine therapy was performed only in a few patients. No consent of patients and their parents for invasive diagnostic procedure was available at that time. Control liver biopsy has not been performed by other researchers[11].

Due to the limited number of data on representative groups of patients, establishing the optimal dose especially for children under the age of 12 years appears still problematic. It appears that increase in daily dose or in frequency of lamivudine administration does not improve the results[12,13,15,26]. The recommended dose of lamivudine results in the same serum concentration of the drug in children as in adults receiving 100 mg per 24 h[26]. The experiences with treatment of chronic hepatitis B in adult patients suggest that this dose of lamivudine seems to be satisfactory.

In our group of patients, 100 mg lamivudine tablets were administered, and the dose per kilogram of body weight varied from 1.3 to 4.1 mg/kg per d, mean 3.0 mg/kg per d. Lamivudine in suspension is not widely available. The average dose of lamivudine in our group is comparable with that recommended by other authors[12,13,15,26].

Based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and monthly laboratory tests during and every 3 mo after the therapy, no particular side-effects were observed in our group of children.

Lebensztejn *et al*[27] reported a case of a child with chronic hepatitis B treated with lamivudine who developed thrombocytopenia and found that withdrawal of lamivudine could normalize platelets count, while reintroduction of the drug results in relapse of thrombocytopenia.

Slight and transient increase of ALT activity during lamivudine therapy (between the 3rd and the 12th mo) was noted in 4 children of our group of patients. YMDD mutation was detected in 2 of them. Termination of lamivudine therapy after 12 mo did not result in any increase of ALT activity or any other symptoms of liver impairment in the follow-up. Some authors have reported the risk of liver dysfunction and even acute liver failure after cessation of lamivudine therapy especially in patients with advanced liver fibrosis or cirrhosis[9]. Advanced fibrosis (stages 3 and 4) could be detected only in 5.1% of children with chronic hepatitis B, which may be the reason for no complications in our group of patients.

Because of economical reasons, only patients with ALT increase were evaluated for YMDD mutation and thus no conclusions about the incidence of YMDD mutation in children with chronic hepatitis B treated with lamivudine can be established. Thus, it can not be ruled out that mutation in the YMDD motif is responsible for the development of resistance to lamivudine in non-responders.

Duration of lamivudine therapy increases not only the rate of positive viral response, but also the risk of YMDD mutations[9], which appears to rise up to 60% in patients treated with lamivudine for more than 4 years[16,24,28]. How-

ever, besides the high percentage of viral mutants, lamivudine is still capable of inducing HBeAg/HBeAb seroconversion and improving histopathological changes within liver tissue in the treated patients[29,30].

In conclusion, one year lamivudine therapy for children with chronic hepatitis B is effective and well tolerated. Seroconversion of HBeAg/HBeAb and SVR are connected with lower pre-treatment serum HBV DNA level.

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RAPID COMMUNICATION

Pegylated IFN-α 2b added to ongoing lamivudine therapy in patients with lamivudine-resistant chronic hepatitis B

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Abstract

AIM: To investigate the role of pegylated-interferon (IFN) α-2b in the management of patients with lamivudine-resistant chronic hepatitis B.

METHODS: Twenty consecutive anti-HBe positive patients were treated with pegylated IFN α-2b (100 μg sc once weekly) for 12 mo. There was no interruption in lamivudine therapy. Hematology, liver biochemistry, serum HBV DNA levels were detected by PCR, and vital signs were also assessed. Liver histology was assessed in some patients at entry and at wk 52 for comparison.

RESULTS: Nine patients (45%) had a partial virological end-treatment response; seven patients (35%) showed complete virological end-treatment response. Eight patients (40%) showed biochemical end-treatment response. There was a trend for higher virological response rates in patients who had previously responded to IFN and relapsed compared to IFN non-responders (four out of seven patients *vs* none out of six patients, respectively; $P = 0.1$). Patients without virological end-treatment response showed significant worsening of fibrosis [median score 2 (range, 1 to 3) *vs* median score 3 (range, 1 to 4)], in the first and second biopsy respectively ($P = 0.014$), whereas necroinflammatory activity was not significantly affected. Patients with complete or partial virological end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 5$). Nevertheless, after 12 mo of follow-up, only one patient (5%) showed sustained virological response and only 2 patients (10%) showed sustained biochemical response. Two patients (10%) discontinued pegylated IFN both after 6 mo of treatment due to flu-like symptoms.

CONCLUSION: Pegylated IFNα-2b, when added to ongoing lamivudine therapy in patients with lamivudine-resistant chronic hepatitis B, induces sustained responses only in a small minority of cases.

Key words: Pegylated interferon; Lamivudine resistance; HBeAg negative chronic hepatitis B; Adefovir

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INTRODUCTION

Lamivudine has been a major breakthrough in the care of patients with chronic hepatitis B (CHB). Nevertheless, almost all patients with HBeAg-negative CHB, which accounts for the majority of patients in Greece, require long-term therapy with lamivudine to maintain a response[1,2]. This strategy is associated with the frequent emergence of viral resistance with reported rates of 10%-27% at 1 year, 40%-56% at 2 years and 67% at 3 years of treatment[3-7]; in a previous study in 77 patients with anti-HBe-positive CHB we have found resistance rates of 1.6% at 9 mo, 3.3% at 12 mo, 12.7% at 15 mo, 15% at 18 mo and 31% at 48 mo[8]. Once lamivudine resistance occurs, patients may experience an attenuation of the initial clinical, virologic and histologic benefits of therapy[9]. The wide use of lamivudine in CHB during the last 5-6 years has resulted in the constant increase of patients with lamivudine-resistant mutants. Their treatment may be the most pressing task for the current anti-HBV strategies in clinical practice[10]. Adefovir dipivoxil is the only approved agent that has been

Table 1 Baseline demographic and clinical features of the study population

No	Sex	Age (yr)	*t* (prior lamivudine therapy) mo	Lamivudine resistance mutation	HBV DNA level (10^3 log_{10} copies/L)	ALT level (times the ULN)
1	M	53	26	L180M + M204V	9.36	1.3
2	M	55	14	L180M + M204I	6.56	6
3	M	63	18	L180M + M204I	7.23	7
4	M	53	25	L180M + M204V	6.68	2.75
5	F	55	19	M204I	6.57	8.5
6	M	63	11	L180M + M204V	6.58	9
7	M	62	10	M204I	8.16	1.3
8	M	47	24	L180M + M204V	7.23	2
9	M	53	18	L180M + M204V	6.86	2.5
10	M	46	16	L180M + M204I	7.98	10
11	M	66	24	L180M + M204V	6.00	1.5
12	F	48	22	L180M + M204I	7.60	3
13	M	54	21	L180M + M204V	9.30	7
14	M	60	41	L180M + M204I	7.51	3.8
15	M	38	29	L180M + M204V	6.26	2.5
16	M	61	22	L180M + M204V	6.57	5
17	M	42	8	M204I	7.43	3.5
18	M	61	25	L180M + M204V	6.98	7
19	M	65	12	L180M + M204I	7.26	5
20	M	54	12	M204I	7.55	2

shown to be effective in this setting, whilst entecavir is also a potential candidate[11-13]. The efficacy of interferon-α (IFN) therapy has not yet been evaluated in any well-designed study in these patients, and therefore no conclusions can be drawn.

IFN-α has a dual mode of action, antiviral via inhibition of viral replication, and immunomodulatory via enhancement of the immunological response of the host against the virus[14]. Pegylation of interferon leads to improved pharmacokinetic and pharmacodynamic profiles, which translated to superior efficacy, compared with conventional, nonpegylated IFN, in the treatment of chronic hepatitis C, and more recently, CHB[15-20]. Two modulations of pegylated IFN (PEG-IFN) are currently being used in clinical practice, namely PEG-IFN-α 2b and PEG-IFN-α 2a. This study was designed to explore the role of PEG-IFN-α 2b in the management of patients with CHB with lamivudine-resistant HBV.

MATERIALS AND METHODS

Subjects

Between November 1999 and February 2003, a total of 20 consecutive anti-HBe positive patients [18 males (90%)], with a median age of 54 (range, 38-66) years, were enrolled in this prospective study. Patients eligible for the study were aged 18 years and older, hepatitis B surface antigen (HBsAg) positive, and receiving ongoing lamivudine therapy for CHB for at least 6 mo at the time of screening; median duration of prior lamivudine therapy was 20 (range, 8-41) mo. All patients were HBeAg negative and antiHBe positive (both at the beginning of prior lamivudine therapy and at the beginning of the present study), genotype D, and had confirmed HBV polymerase gene mutation within the YMDD motif by DNA sequencing (Trugene HBV genotyping, Visible Genetics Inc); lamivudine resistance mutations are shown in Table 1. Patients were required to have a screening HBV DNA level $>10^9$ copies/L (Amplicor HBV-DNA Monitor Test; Roche Diagnostics, Branchburg, NJ, USA, with a sensitivity of 400×10^3 copies/L) as well as elevated serum alanine aminotransferase (ALT) levels >1.2 times the upper limit of normal (ULN) on at least 2 occasions at least 1 mo apart within the preceding 6 mo. The exclusion criteria are as follows: absolute neutrophil count $\leqslant 10^9$ cells/L; hemoglobin $\leqslant 100$ or $\leqslant 90$ g/L (males or females, respectively); platelet count $< 50 \times 10^9$/L; prior treatment with interferon or other immunomodulatory therapies within the 6 mo preceding study screening; serious concurrent medical conditions, including other concurrent liver diseases; coinfection with hepatitis C virus or hepatitis delta virus or human immunodeficiency virus; current alcohol or substance use; and pregnancy and/or lactation. None of the patients had liver cirrhosis.

Amongst the 20 patients enrolled, 7 (35%) were naive to IFN and 13 (65%) had been previously treated with IFN 5 MU sc three times weekly for at least 12 mo (before receiving lamivudine); six of the latter had shown no response and seven had responded to IFN (i.e. had shown both reduction in serum HBV DNA level to $<10^8$ copies/L and normalization of ALT level at the end of IFN administration) but relapsed after discontinuing therapy.

Methods

Patients were treated with pegylated interferon α-2b (100 μg sc once weekly) for 12 mo. There was no interruption in lamivudine therapy, even after the cessation of PEG-IFN-α 2b. Patients were evaluated every month. At each visit, any untoward medical occurrences, regardless of causality, were recorded as adverse events. Hematology, liver biochemistry, serum HBV DNA levels, and vital signs were also assessed. Liver histology was assessed in 13 patients at entry and at wk 52 for histological comparison; the rest of the patients denied a liver biopsy. A single pathologist, who was blinded to the sequence of the biopsies, evaluated all

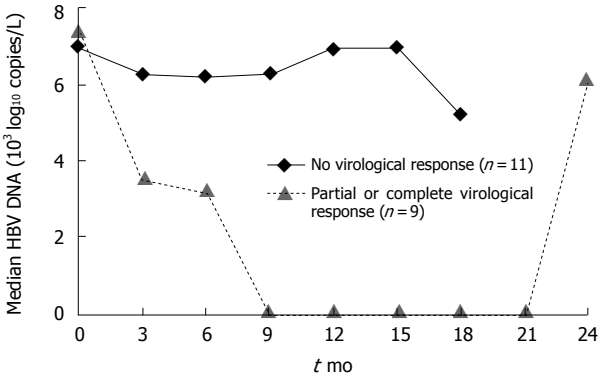

Figure 1 Median HBV DNA titers during PEG-IFN-α 2b therapy and during follow-up.

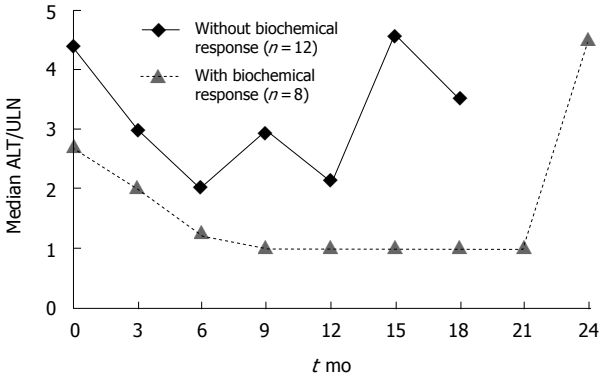

Figure 2 Median ALT values, expressed as multiples of ULN, during PEG-IFN-α 2b therapy and during follow-up.

specimens. Inflammation and fibrosis were each classified into 4 stages according to the Scheuer system.

After the cessation of PEG-IFN-α 2b, adefovir dipivoxil, 10 mg once daily, was given to patients who did not show a reduction in serum HBV DNA level to below 10^8 copies/L at wk 52 and to patients who demonstrated virologic breakthrough during follow-up (defined as reappearance of serum HBV DNA on two consecutive occasions, at least 3 mo apart, after its initial disappearance). The primary end point of the study was a reduction in serum HBV DNA level (partial virological end-treatment response). This was assessed as the proportion of patients with HBV DNA level $<10^8$ copies/L at wk 52. Secondary end points included the proportion of patients with undetectable HBV DNA at wk 52 (complete virological end-treatment response), the percentage of patients with normalization of ALT level at wk 52 (biochemical end-treatment response) and undetectable HBV DNA by PCR and normalization of ALT level after 12 mo of follow-up (virological and biochemical sustained response, respectively).

The study was approved by the ethics committee of our institution, and all patients provided written informed consent before screening. The study was performed in accordance with the principles of the Declaration of Helsinki.

Statistical analysis

All data were analyzed using the statistical package SPSS (version 10.0; SPSS Inc., Chicago, IL). The population analyzed included all patients who received at least one dose of study medication. The Mann-Whitney and Chi-square tests were used for comparisons of quantitative and qualitative variables respectively. The Wilcoxon Signed rank test was used to test the effect of PEG-IFN-α 2b on histological findings in the patients who underwent paired liver biopsies. In all cases, a 2-tailed *P* value less than 0.05 was considered statistically significant.

RESULTS

Baseline demographic and clinical features of the study population are presented in detail in Table 1. At baseline, median HBV DNA level was 7230 log_{10} copies/L (range, 6 000-9 360 log_{10} copies/L) and median ALT level was 3.5 (range, 1.3-10) times the ULN. Changes in median HBV DNA titers during PEG-IFN-α 2b therapy are shown in Figure 1. After 52 wk of treatment, 9 patients (45%) had a partial virological end-treatment response. The median change from baseline in serum HBV DNA levels was -7000 (range, -9 400 to 7 600) log_{10} copies/L. Seven patients (35%) showed complete virological end-treatment response. In these patients HBV DNA became undetectable after a median of 9 (range, 3 to 12) mo. Eight patients (40%) showed biochemical end-treatment response. In these patients normalization of ALT levels occurred after a median time of 9 (range, 3 to 12) mo (Figure 2). Overall, serum ALT level decreased over 52 wk in 16 patients (80%). The median ALT level at baseline was 3.5 times the ULN; by wk 52, this had declined to 2.2 times the ULN.

None of the baseline demographic and clinical features predicted virological (partial or complete) or biochemical end-treatment responses. Nevertheless, it should be noted that none of the six patients who had not responded to prior IFN treatment showed complete virological end-treatment response, while four out of the seven patients who had relapsed after an initial response to IFN showed complete virological end-treatment response ($P = 0.1$). The findings in the 13 patients in whom biopsies were performed are shown in Table 2 and in Figure 3. Patients without virological end-treatment response showed significant worsening of fibrosis ($P = 0.014$) in the second biopsy, whereas necroinflammatory activity was not significantly affected. Patients with complete or partial virological end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 5$). Likewise, patients without biochemical end-treatment response showed significant worsening of fibrosis ($P = 0.014$) in the second biopsy, whereas necroinflammatory activity was not significantly affected. Also, patients with biochemical end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 3$).

During follow up, HBV-DNA reappeared in six out of the seven patients who had shown complete virological end-treatment response, giving an overall sustained virological response rate of 5%. The median time to HBV-DNA re-emergence was 5 (range, 1 to 12) mo. Changes in median

Table 2 Histological findings in 13 patients with biopsy performed [median (range)]

	Baseline biopsy		End of treatment biopsy	
Virological end-treatment response	Necroinflammatory activity	Fibrosis	Necroinflammatory activity	Fibrosis
None (n=8)	3 (1-3)	2 (1-3)	2 (1-4)	3 (1-4)
Partial (n=2)	2.5 (2-3)	2.5 (2-3)	2.5 (2-3)	2.5 (2-3)
Complete (n=3)	2 (2-3)	2 (2-2)	1 (1-2)	1 (1-2)

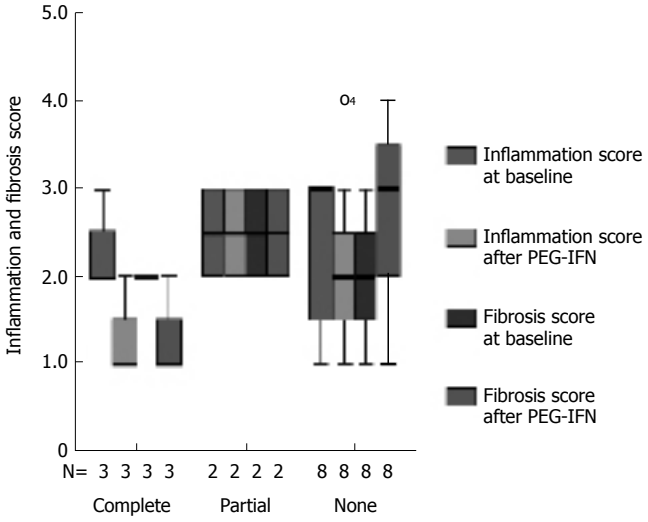

Figure 3 Changes in median inflammation and fibrosis scores according to end of treatment virological response.

HBV DNA titers during follow-up are shown in Figure 1 (patients' data are censored at the time of commencement of treatment with adefovir dipivoxil). Two patients (10%) had a sustained biochemical response while in the remaining 6 patients with biochemical end-treatment response, ALT became abnormal after a median of 4 (range, 1 to 12) mo. Changes in median ALT levels during follow-up are shown in Figure 2 (patients' data are censored at the time of commencement of treatment with adefovir dipivoxil). Two patients (10%) discontinued PEG-IFN both after 6 mo of treatment and both due to flu-like symptoms (fatigue, low-grade fever, arthralgia and headache). Neither of these patients showed virological or biochemical end-treatment response; in fact, one of them showed a rise in HBV-DNA levels and both showed a rise in ALT levels at 52 wk. PEG-IFN was well-tolerated in all other patients and none of them required dose reduction or interruption of therapy. There were instances of hepatic decompensation during the study.

DISCUSSION

Richman[21] has recently defined an antiviral drug as one that selects for resistance. Antiviral drug resistance depends on the viral mutation frequency, intrinsic mutability of the antiviral target site, the selective pressure exerted by the drug, and the magnitude and rate of virus replication. In particular, lamivudine resistance is due to mutations within the YMDD motif in the major catalytic region C of the HBV polymerase gene[22]. Viral resistance is clinically expressed by the virological breakthrough phenomenon, defined as the reappearance of serum HBV DNA after an initial clearance of viraemia despite the continuation of therapy[10]. The emergence of resistance has a negative impact on the efficacy of therapy in CHB patients, since virological breakthroughs are almost invariably followed by increasing viraemia levels, culminating in biochemical breakthroughs, which ultimately have an adverse effect on liver histology[6].

It is clear that the possible adverse effects of YMDD mutants do cast a concern. Rescue therapies for patients with worsening liver disease caused by lamivudine-resistant mutants are being evaluated. Until recently, treatment options for these patients have been limited to continuation or cessation of lamivudine therapy. Continuation of lamivudine aims to further suppress or to prevent the return of wild-type HBV which is more replicative competent than the YMDD mutant[23]. However, this strategy seems ineffective. Lamivudine withdrawal results in re-emergence of wild-type HBV within 3–4 mo[24]. Therefore, acute exacerbations of liver disease might ensue and could, although uncommon, result in hepatic decompensation or acute liver failure[25]. Adefovir dipivoxil effectively inhibits replication of YMDD mutants resistant to lamivudine and hence averts the resultant disease; entecavir has also shown promising results[11-13]. Nevertheless, neither of these novel nucleoside analogues was licensed in Greece during the study period.

There is a paucity of data regarding the role of IFN in the treatment of lamivudine-resistant HBV. Interferon-α has multiple sites of action in the viral life cycle and may be effective against lamivudine-resistant virus[26]. Recently, lamivudine was found to restore cytotoxic T-cell responses in patients with CHB, and, therefore, it may augment the immunomodulatory activity of IFN[27]. Hence, there would be a rationale in treating patients with lamivudine-resistant HBV mutants with IFN. Pegylation is the attachment of a polyethylene glycol (PEG) molecule to the base IFN molecule resulting in effective concentrations of IFN throughout the dosing interval and substantially reduced peak-to-trough ratio, in contrast to conventional IFN, which yields only intermittent drug exposure; pegylation also allows for once weekly frequency of administration[14]. Pegylated IFN has been shown to be highly active against wild-type HBV infection, both HBeAg-positive[15,17-19] and HBeAg-negative[20]. Complete virological end-treatment response was achieved by 35% of our patients. Therefore, this study confirms that PEG-IFN-α 2b is also active against lamivudine-resistant HBV. Of note, complete virological end-treatment response was achieved by 63% of patients with wild-type HBeAg-negative CHB in a recent landmark study[20]. The small number of patients included in the present report might *per se* preclude direct comparisons of efficacy between these two studies; however, different patients' characteristics might have also accounted for the apparently inferior results of PEG-IFN therapy in our study. Even though no pretreatment factor

has been found to be reliably associated with a response to IFN in HBeAg-negative CHB[1], it must be mentioned that our patients were older and had higher ALT and lower HBV-DNA levels at baseline than the patients in the aforementioned study[20]. Of course, it is also possible that PEG-IFN might not be as effective in lamivudine-resistant as in wild-type HBV strains, but this has to be investigated further in large-scale studies.

Patients with HBeAg-negative CHB receiving IFN retreatment respond as well as naive ones, irrespective of the outcome of the initial treatment[28]. In accordance with this, in our study, prior IFN treatment, as well as its outcome, was not associated with the efficacy of PEG-IFN-α 2b. Nevertheless, this could be attributed to the limited number of patients studied, since there was a trend for higher virological response rates in patients who had previously responded to IFN and relapsed compared to IFN non-responders ($P = 0.1$).

In patients with HBeAg-negative CHB, the 12 mo sustained response rates to IFN treatment vary from 10% to 47% (average 24%)[1]; sustained 6 mo biochemical and complete virological response rates with PEG-IFN rise up to 59% and 19%, respectively[20]. The low percentage of sustained biochemical and complete virological response (10% and 5% respectively) in our patients is of concern and renders PEG-IFN-α 2b rather unattractive for patients with lamivudine-resistant CHB. Nevertheless, the already mentioned differences in patients' characteristics between studies and the inherent limitation of the small number of patients included in our report might have contributed to these discrepant findings. Furthermore, it must be pointed out that the time point of evaluation of the sustained response in our study was at 12 mo after treatment completion compared to 6 mo in the wild-type HBV study[20], and this should be taken into account when comparing our results with the latter ones. However, the issue of differing activity of IFN in lamivudine-resistant compared to wild-type HBV strains definitely needs to be addressed.

In conclusion, this study shows that, 52 wk of treatment with PEG-IFN-α 2b, when added to ongoing lamivudine therapy in patients with lamivudine-resistant CHB, induces sustained responses only in a small minority of cases. Therefore, other treatment strategies should be considered for these patients, possibly including more prolonged or earlier (when the viral load is less than 10^6 copies/L) administration of PEG-IFN-α 2b.

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RAPID COMMUNICATION

One-step palliative treatment method for obstructive jaundice caused by unresectable malignancies by percutaneous transhepatic insertion of an expandable metallic stent

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Abstract

AIM: To describe a simple one-step method involving percutaneous transhepatic insertion of an expandable metal stent (EMS) used in the treatment of obstructive jaundice caused by unresectable malignancies.

METHODS: Fourteen patients diagnosed with obstructive jaundice due to unresectable malignancies were included in the study. The malignancies in these patients were a result of very advanced carcinoma or old age. Percutaneous transhepatic cholangiography was performed under ultrasonographic guidance. After a catheter with an inner metallic guide was advanced into the duodenum, an EMS was placed in the common bile duct, between a point 1 cm beyond the papilla of Vater and the entrance to the hepatic hilum. In cases where it was difficult to span the distance using just a single EMS, an additional stent was positioned. A drainage catheter was left in place to act as a hemostat. The catheter was removed after resolution of cholestasis and stent patency was confirmed 2 or 3 d post-procedure.

RESULTS: One-step insertion of the EMS was achieved in all patients with a procedure mean time of 24.4 min. Out of the patients who required 2 EMS, 4 needed a procedure time exceeding 30 min. The mean time for removal of the catheter post-procedure was 2.3 d. All patients died of malignancy with a mean follow-up time of 7.8 mo. No stent-related complication or stent obstruction was encountered.

CONCLUSIONS: One-step percutaneous transhepatic insertion of EMS is a simple procedure for resolving biliary obstruction and can effectively improve the patient's quality of life.

Key words: Expandable metallic stent; Bile duct carcinoma; Gall bladder carcinoma; Pancreatic carcinoma; Gastric carcinoma; Obstructive jaundice

Yoshida H, Mamada Y, Taniai N, Mizuguchi Y, Shimizu T, Yokomuro S, Aimoto T, Nakamura Y, Uchida E, Arima Y, Watanabe M, Uchida E, Tajiri T. One-step palliative treatment method for obstructive jaundice caused by unresectable malignancies by percutaneous transhepatic insertion of an expandable metallic stent. *World J Gastroenterol* 2006; 12(15): 2423-2426

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INTRODUCTION

The incidence of biliary obstruction resulting from malignancies is increasing. As operative techniques and diagnostic imaging have advanced, more and more patients undergo resection. However, in cases where operation is not possible, prognosis remains poor often because of the presence of obstructive jaundice.

Palliative treatment with a biliary stent is carried out in patients with inoperable malignancies in order to relieve symptoms related to obstructive jaundice, prevent cholangitis and prolong survival. In addition, the biliary insertion procedure only requires a short time of hospitalization, which is especially important for patients with unresectable malignancies because of their poor prognosis.

When first introduced, stent insertion is performed using polyethylene endoprosthese. However, the expandable metallic stent (EMS) has also been available for a number of years [1, 2]. EMSs have advantages over plastic stents in that they can be introduced through a smaller delivery catheter, have a larger inner diameter and remain fixed in position after

release [3-6].

It was previously reported that one-step method of percutaneous transhepatic insertion of EMSs can be used for obstructive jaundice due to unresectable common bile duct carcinoma[7], further supporting the one-step method of percutaneous transhepatic insertion of EMS to treat obstructive jaundice caused by unresectable malignancies.

MATERIALS AND METHODS

Patients

This study comprised 14 patients with obstructive jaundice caused by unresectable malignancies, who were admitted into Nippon Medical School or Uchida Hospital from 2002 to 2004. Of the 14 patients (6 men and 8 women), 1 suffered from gastric carcinoma, 3 from recurrence of gastric carcinoma, 4 from pancreas carcinoma, 3 from bile duct carcinoma, 1 from gall bladder carcinoma, and 2 from recurrence of gall bladder carcinoma. The age distribution of the patients ranged between 65-90 years, with a mean age of 77.1 years.

The diagnosis was confirmed by ultrasonography and computed tomography. All patients were diagnosed before cholangiography as having obstructive jaundice caused by unresectable malignancies as a result of very advanced carcinoma or old age.

This study was performed according to the principles of the Declaration of Helsinki, and informed consent was obtained from the patients and/or their families.

Methods

The procedure was performed single-handedly (Hiroshi Yoshida). The portion of the procedure was consistent for all the patients. Following pre-medication with an intravenous injection of pentazocine (15 mg) and hydroxyzine (25 mg), the patient was given a local anesthetic consisting of 1% xylocaine. The appropriate intrahepatic bile duct of the lateral segment or right lobe of the liver was punctured with a sheath needle (19 gauge × 150 mm; Hakko, Tokyo, Japan) under ultrasonographic guidance. Percutaneous transhepatic cholangiography was then performed (Figure 1A).

The bile duct obstruction was cleared using a guide wire (RADIFOCUS GUIDE WIRE M, 0.035 inch × 150 cm; TERUMO, Tokyo, Japan). After a catheter with an inner metallic guide (EV drainage catheter, 17 gauge × 270 mm; Hakko, Tokyo, Japan) was advanced into the duodenum, a contrast material was injected to determine the overall length of stenosis (Figure 1B). The guide wire was reinserted and the EV drainage catheter was substituted with an EMS (SMART stent, 10 mm in diameter × 80 mm long; Cordis Endovascular, Warren, NJ) system. In cases where it was difficult to advance the EMS system into the duodenum, a dilatation catheter (PTCS catheter, 9 Fr × 60 cm; Sumitomo Bakelite, Akita, Japan) was inserted prior to insertion of the EMS system. The EMS was then placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the entrance to the hepatic hilum, irrespective of the size of stenotic lesion present (Figure 2A). Where it was difficult to span the distance between the above points with a single EMS, an additional EMS SMART stent (10 mm in diameter × 60 mm long) was inserted connecting lengthways to the first stent. The EV drainage catheter or PTCS catheter was left in place to act as a hemostat at the site of insertion to the liver (Figure 2B). The patient received antibiotics following the stent insertion procedure. As an additional measure, saline was injected daily into the catheter to flush out the bile duct. The catheter was removed after resolution of cholestasis and stent patency was confirmed 2 or 3 d post-procedure.

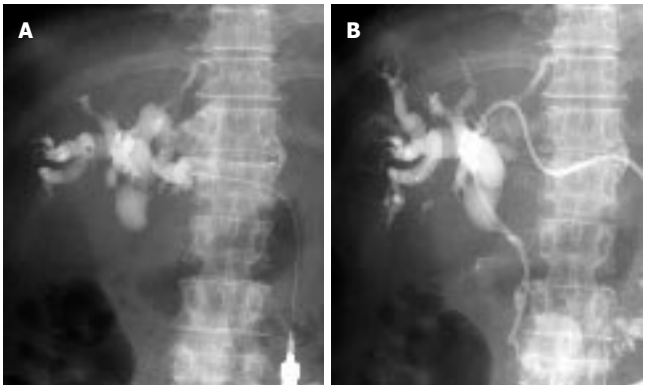

Figure 1 Insertion procedure of EMS (**A**) and the overall length of stenosis (**B**). Appropriate intrahepatic bile duct of the lateral segment of the liver was punctured with a sheath needle under ultrasonographic guidance. Percutaneous transhepatic cholangiography was performed. The bile duct obstruction was cleared using a guide wire. After a catheter with an inner metallic guide was advanced into the duodenum, contrast material was injected to determine the overall length of stenosis.

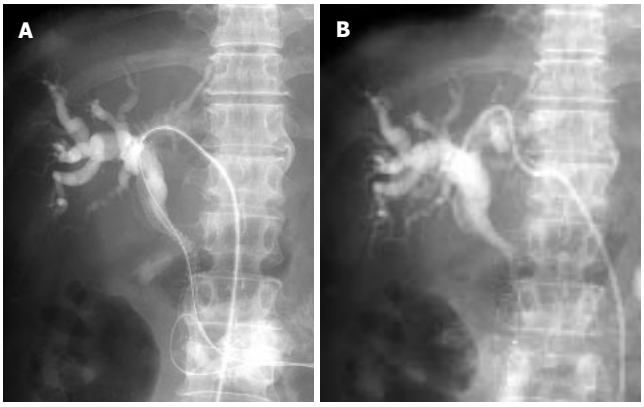

Figure 2 Placement of EMS (**A**) and EV drainage(**B**). The guide wire was reinserted and the EV drainage catheter was substituted with an EMS system. The EMS was placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the hepatic hilum, irrespective of the size of the stenosis lesion present. The EV drainage catheter was left in place to act as a hemostat at the insertion site of the liver.

RESULTS

One-step insertion of the EMS was achieved in all patients. The procedure time ranged between 15-42 min with a mean time of 24.4 min. A single EMS was required in 7 patients, while two EMSs were needed in the other 7.

Table 1 Outcome after insertion EMS

Patient No.	Age	Sex	Disease	Procedure time (min)	No.of EMS	Days[2]	Outcome
1	90	F	Bile duct ca.	32	2[1]	3	death after 8 mo
2	72	F	Bile duct ca.	35	2[1]	2	death after 5 mo
3	87	F	Bile duct ca.	20	2	3	death after 7 mo
4	72	M	Gastric ca.	31	2	2	death after 5 mo
5	67	M	Recurrence of gastric ca.	16	1	2	death after 4 mo
6	66	F	Recurrence of gastric ca.	15	1	2	death after 12 mo
7	90	F	Recurrence of gastric ca.	21	2	3	death after 10 mo
8	72	M	GB ca.	42	2	2	death after 3 mo
9	84	F	Recurrence of GB ca.	20	1	3	death after 17 mo
10	65	F	Recurrence of GB ca.	28	1	2	death after 11 mo
11	82	M	Pancreas ca.	20	1	2	death after 8 mo
12	76	M	Pancreas ca.	18	1	2	death after 5 mo
13	72	M	Pancreas ca.	22	1	2	death after 6 mo
14	84	F	Pancreas ca.	22	2	2	death after 6 mo
Mean	77.1			24.4	1.5	2.3	7.8

EMS:expandable metallic setent ca:carcinoma. GB:gall bladder. 1: Dilation catheter was used before insertion of EMS. 2: Postprocedure d of catheter removed.

In 2 patients, a PTCS catheter was used because of prior difficulty in inserting the EMS system. Out of the patients who required 2 EMSs, 4 of these needed a procedure time exceeding 30 min.

After successful insertion of the stent, cholestasis rapidly resolved itself in all the patients. The catheter was removed after a mean post-procedure time of 2.3 d. All patients died of malignancy with a mean follow-up time of 7.8 mo. No stent-related complication or stent obstruction was encountered (Table 1).

DISCUSSION

Long-term survival is poor in patients with malignant bile duct obstruction and in those who are not candidates for surgical resection. The objective of palliation with a biliary stent is to relieve symptoms related to obstructive jaundice, prevent cholangitis and prolong survival. Stenting has also been shown to improve patient quality of life[8].

Since the development of suitable metallic stents, a debate has arisen regarding when to use a metallic stent in preference to a plastic one. Randomized studies comparing metallic and plastic endoprostheses demonstrated that the metallic stent is associated with a lower incidence of complications, remains patent longer and is more cost-effective, although it is initially more expensive[5,6, 9-11].

Complications arising from metal stent placement for malignant bile duct obstruction, including tumor ingrowth or overgrowth[4,12], viscus perforation [13, 14], fracture[15], and stent migration[16] have been reported. The early occlusion rate ranges between 7%-42% and the late occlusion rate ranges between 12%-38%, with a mean time to stent failure of 6-9 mo[5,6,10,11,17] . In this study, no EMS-related complication or obstruction was encountered. A possible reason is that all the patients suffered from very advanced carcinoma and could not undergo surgery before cholangiography. Therefore, the prognosis of the patients was poor and patients died due to malignancy after just 7.8 mo. A further possible reason is that the EMS was placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the hepatic hilum, irrespective of the size of stenotic lesion, therefore the stenosis was not completely covered to avoid overgrowth. In addition, saline was injected into the catheter on a daily basis to flush out the bile duct and stent.

In this study, percutaneous transhepatic insertion of the EMS was conducted. A different approach is to insert the EMS endoscopically. Recently, it was reported that the EMS can be inserted from the papilla of Vater under endoscope[18-20]. The success rate is below 100% and the time of procedure is relatively long[18-21]. This is because dilation of the stenotic lesion and control of a long EMS system are difficult in this approach. For high-risk patients with very advanced carcinoma or old age, it is imperative to keep operative procedures as straightforward and short as possible. In the endoscopic approach, the lengthy time of procedure is too invasive to be carried out on high-risk patients. With the percutaneous transhepatic insertion in this study, the distribution of the procedure time ranged between 15 - 42 min, with a mean time of 24.4 min. The EMS insertion could be performed single-handedly, easily, and quickly.

A disadvantage of the percutaneous transhepatic approach is that the catheter must be left in place to act as a hemostat at the insertion site of the liver. However, this is also a benefit as the catheter could then be used to confirm the stent patency and to flush the EMS free from coagula or debris for 2 or 3 d post-procedure.

In conclusion, the one-step percutaneous transhepatic insertion of EMS is a simple procedure for resolving biliary obstruction and can effectively improve the patient's quality of life.

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RAPID COMMUNICATION

Effect of oral garlic on arterial oxygen pressure in children with hepatopulmonary syndrome

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Abstract

AIM: To study the effect of oral garlic on arterial oxygen pressure in children with hepatopulmonary syndrome.

METHODS: Garlic powder in a capsule form was given to 15 children with hepatopulmonary syndrome (confirmed by contrast echocardiography) at the dosage of 1 g/1.73 m^2 per day. Patients were evaluated clinically and by arterial blood gas every four weeks.

RESULTS: The garlic capsule was administered to 15 patients with hepatopulmonary syndrome. There were 10 boys and 5 girls with a mean age of 9.4 ± 3.9 years. The underlying problems were biliary tract atresia (4 patients), autoimmune hepatitis (4 patients), cryptogenic cirrhosis (4 patients) and presinusoidal portal hypertension (3 patients). Eight patients (53.3%) showed an increase of 10 mmHg in their mean arterial oxygen pressure. The baseline PaO_2 was 65.6 ± 12.1 mmHg in the responder group and 47.1 ± 11.2 mmHg in non-responder group. At the end of treatment the mean PaO_2 in responders and non-responders was 92.2 ± 7.75 mmHg and 47.5 ± 11.87 mmHg, respectively ($P < 0.01$).

CONCLUSION: Garlic may increase oxygenation and improve dyspnea in children with hepatopulmonary syndrome.

Key Words: Hepatopulmonary syndrome; Garlic; Arterial oxygen pressure; Pediatric

INTRODUCTION

In 1995, Lange *et al* [1] introduced the term "hepatopulmonary syndrome" for those patients with pulmonary vasodilatation associated with chronic liver disease and cyanosis. Hepatopulmonary syndrome is defined by an arterial oxygen pressure < 70 mmHg in room air. In adults, the prevalence is 5%-29% and the overall mortality is 41% in hospitalized patients. In children, the prevalence ranges from 0.5% in those with portal vein obstruction to 20% in children with biliary atresia and polysplenia syndrome. In other causes of childhood cirrhosis, the prevalence is 2% -4%. Hepatopulmonary syndrome has been described in children as young as 6 months. Generally, hepatopulmonary syndrome is seen in chronic liver disease, but it has also been described in acute liver failure and extrahepatic portal venous obstruction [2].

A right to left pulmonary shunt due to intrapulmonary vascular dilation characterizes this syndrome. Blood flowing through the dilated capillaries is less exposed to oxygen contained in the alveoli, resulting in a ventilation-perfusion mismatch [3-6]. In animal models, elevated levels of nitric oxide have been detected in the lung homogenates of animals with the clinical features of hepatopulmonary syndrome. This finding appears to be supported by reports documenting increased exhaled nitric oxide concentration in patients with hepatopulmonary syndrome [7, 8].

The best method to evaluate the shunt in hepatopulmonary syndrome is the contrast-enhanced echocardiogram [1, 2, 9, 10]. Technetium 99m-labeled macroaggregated albumin scanning is a second method of detecting intrapulmonary vascular dilatations. Pulmonary arteriography can suggest two angiographic patterns [1]. Schneck *et al* [13] have reported the beneficial effect of methylene blue on this syndrome [13]. The transjugular intrahepatic portosystemic shunt (TIPS) [14], cavoplasty [15], octreotide [16], indomethacin [17, 18] and embolization [19] in the management of this syndrome have been assessed.

In 1992, clinical improvement in a case report was

Table 1 Baseline characteristics of children with hepatopulmonary syndrome

	Age (yr)	Gender	Type of disease	Duration (yr)	Child score	Dyspnea
Non-responders	14	M	Cryptogenic cirrhosis	10	C	+
	10	M	AIH	5	B	-
	6	M	Biliary atresia	6	C	+
	3	F	Biliary atresia	3	C	+
	13	M	Presinusoidal	10	A	+
	12	F	Presinusoidal	9	C	+
	9	M	Biliary atresia	9	C	+
Responders	7	F	Cryptogenic	4	B	+
	12	M	Biliary atresia	11	C	+
	3	F	AIH	1	A	+
	14	M	Cryptogenic cirrhosis	9	B	+
	13	M	Presinusoidal	8	A	+
	4	M	AIH	1	A	-
	11	M	Cryptogenic cirrhosis	5	B	-
	11	F	AIH	5	A	-

AIH: Autoimmune hepatitis.

achieved following the use of garlic [20]. In a study by Abrams *et al* [21] the use of garlic in adults in treatment of this syndrome was evaluated and garlic was found to be an effective therapy for this syndrome especially in young groups. Ku *et al* [22] revealed that active garlic metabolites are capable of pulmonary vasodilatation in rats. In 1997 Battaglia *et al* [23] reported an improvement in right to left shunt and arterio-alveolar gradient in such patients after liver transplantation. Further studies have confirmed the beneficial effect of liver transplantation on this syndrome [24, 25]. In our country, liver transplantation has a long waiting list. If the oral garlic can improve the arterial oxygenation and dyspnea, it may represent a palliative therapy for patients with hepatopulmonary syndrome awaiting liver transplantation.

MATERIALS AND METHODS

Patients

Fifteen patients (aged 6 months - 14 years) suffering from chronic liver disease, portal hypertension or cirrhosis and intrapulmonary shunt which were confirmed by saline contrast echocardiography were enrolled in this pilot study. Patients with congenital heart diseases, acute and chronic lung diseases and symptoms of severe gastritis, were excluded from the study. This study was performed at Children's Medical Center Hospital of Tehran University from 2002-2003.

Methods

Arterial blood gases were obtained from the radial artery in a sitting position at the same time and in the same room. Echocardiography with saline contrast was performed as previously described [13]. The presence of air bubbles in the left heart between 3 and 6 cardiac contractions indicated intrapulmonary shunt. Garlic was administered to the patients at the dose of 0.5-2 g/1.73 m^2 per day. We obtained a special kind of dried garlic (Hamedan city garlic), which was prepared in the form of 250 mg capsules by the hospital pharmacy.

The parents were asked to add the capsule or its content to their food once or twice a day at the recommended dose. We evaluated the patients every month. Clinical symptoms and signs, probable drug complication, and patient's compliance to the drug were asked in each visit. Arterial blood gas (ABG) was taken in the same condition as mentioned above. During the study we provided some facilities such as free contact if needed.

Response to garlic was defined as a 10 mmHg increment in the PaO_2 or when PaO_2 was more than 70 mmHg. Baseline and monthly data between responders and non-responders were analyzed statistically.

The Ethics Committee of Tehran University approved the protocol. The parents of the children gave their informed consent.

Stastistical analysis

The results were expressed as mean ± SD. Differences between two groups were evaluated by Fisher's exact test, Mann Whitney U and Wilcoxson rank-sum tests. $P < 0.05$ was considered statistically significant.

RESULTS

The baseline data of patients are summarized in Table 1. Fifteen patients with hepatopulmonary syndrome were treated with garlic. One child expired after one month of treatment.

At the end of the first month arterial O_2 pressure was increased significantly ($P = 0.004$). The mean of arterial O_2

Table 2 PaO_2 values of responders and non-responders in relation to dyspnea after treatment

Responders	Baseline	1st mo	2nd mo	3rd mo	4th mo	5th mo	Dypnea after Rx
1	66	85	90	90	90	95	Improved
2	70	86	87	90	99	99	Worsened
3	74	83	85	90	90	95	Improved
4	59	79	85	95	99	99	Improved
5	43	77	79	80	80	80	Improved
6	63	85	95	95	95	95	[a]
7	85	91	90	92	91	95	[a]
8	65	69	76	84	82	80	[a]
Non-responders							
1	34	33	32	35	40	40	No change
2	65	70	71	70	70	69	[a]
3	51	55	54	55	40	40	Worsened
4	57	58	.	.	.	.	Worsened
5	37	42	40	39	45	47	No change
6	45	49	51	54	50	52	No change
7	40	35	37	41	39	37	Worsened

[a]No dyspnea before and after treatment.

Table 3 PaO_2 values after oral garlic administration (mean ± SD, n = 15)

	Baseline	1st mo	2nd mo	3rd mo	4th mo	5th mo
Non-responders	47.1 ± 11.2	48.8 ± 13.2	47.5 ± 14.2	49 ± 13.1	47.3 ± 11.8	47.5 ± 11.8
Responders[b]	65.6 ± 12.1	82 ± 6.8	85.8 ± 6.1	89.5 ± 5.1	90.7 ± 7	92.2 ± 7.7[d]
Total	57 ± 14.7	66.5 ± 19.7	69.4 ± 22	72.1 ± 22.6	72.1 ± 24	73 ± 24.7[d]

[b]P <0.01 *vs* non-responders, [d]P <0.01 *vs* baseline.

pressure increment at the end of the second month was higher than 10 mmHg and over 70 mmHg at the end of the third month. At the end of the fifth month, the mean increment in the arterial O_2 pressure was 15 mmHg (Table 2).

Eight patients (53.3%) were considered as responders. The baseline PaO_2 was 65.6 ± 12.1 mmHg in the responder group and 47.1 ± 11.2 mmHg in non-responder group. There was a statistically significant difference in the initial O_2 pressure between the responders and non-responders (P = 0.009). At the end of treatment the mean PaO_2 in responders and non-responders was 92.2 ± 7.75 mmHg and 47.5 ± 11.87 mmHg, respectively (Table 3).

Dyspnea (including shortness of breath at rest or exercise, orthopnea and platypnea) occurred in 5 responders and 6 non-responders. Four out of these 11 patients had their dyspnea improved after treatment (Table 2).

According to the Child score, 5 patients (33.3%) were in group A, 4 (26.6%) in group B, and 6 (40%) in group C. The mean of arterial oxygen pressure in groups A, B and C, at the beginning of therapy was 56.4 ± 15.6 mmHg, 68.7 ± 11.2 mmHg and 49.6 ± 12.8 mmHg, respectively. At the end of therapy the mean PaO_2 in A, B and C groups was 79.4 ± 19.6 mmHg, 88.2 ± 13.3 mmHg and 53.6 ± 26 mmHg, respectively. No statistical difference in the mean PaO_2 was noted between groups A, B, and C.

DISCUSSION

The role of garlic in the management of hepatopulmonary syndrome is controversial. In 1992, clinical improvement was reported following the use of garlic [20]. In 1998 Abrams *et al* [21] studied the effects of garlic on hepatopulmonary syndrome in adults [21]. Chan *et al* [26] showed that garlic has no effect on the improvement of PaO_2 and hypoxemia as well as clinical status, suggesting that deteriorating oxygenation in patients with chronic liver disease may be an indication for liver transplantation. Abrams *et al* [21] showed that 40% of patients have at least an increase of 10 mmHg in their arterial oxygen pressure after treatment with garlic. They also studied the dyspnea index in these patients and reported that all patients responding to therapy have an improvement in dyspnea [21]. In the present study, 53.3% of patients following the use of garlic had an arterial oxygen pressure > 70 mmHg or an increment > 10 mmHg. Four out of the 8 patients responding to the therapy had an improvement in dyspnea, but non-responders did not show any improvement in their respiratory symptoms.

It was reported that the clinical response is better in younger patients [21]. The results of the present study are consistent with those of Abrams *et al* [21]. We showed an improvement rate of 53.4% in children and Abrames *et al* showed 40% in adults. Nevertheless, no statistically significant differences were noted between responders and non-responders with respect to age.

It is interesting to note that responders had a higher baseline PaO_2, suggesting that non-responders have a severe and profound disease and that medical management can be effective when baseline PaO_2 is higher. Since TC-99 macro-aggregated albumin scan was not performed in the present study, there was a possibility of selection bias, because anatomic shunt failing to respond to medical therapy was not excluded.

The exact mechanism of garlic therapy for hepatopulmonary syndrome is not known. Garlic appears to cause pulmonary vasodilatation [22] and increases the rate of NO synthesis [25, 27,28]. Thus one can conclude that garlic worsens hepatopulmonary syndrome. Garlic causes reduced NO synthesis in macrophages, resulting in reduced concentration in hypoxic tissue [29].

Intrapulmonary vasodilatation occurs primarily in the bases of the lungs, resulting in significant V/Q mismatch in this region. Abrams *et al* [21] speculated that if garlic results in uniform vasodilatation throughout the lung, then a redistribution of pulmonary blood flow to apical and mid lung fields could significantly improve V/Q ratios in these regions. Therefore, garlic may improve V/Q and reduce NO synthesis in the lung bases. Also garlic is known as a hepatoprotective agent and protects liver against tissue and chemical injures. If this is the case, it is probable that garlic might improve liver function and hepatopulmonary syndrome. Garlic might also reduce portal hypertension, indirectly resulting in an improvement in hepatopulmonary syndrome. Additionally, Allicin, Ajoene, and diallyl sulfur may be beneficial for hepatopulmonary syndrome treatment. These ingredients are present in dried garlic in sufficient amount [22]. However, the amounts of these ingredients vary depending on different types of garlic [29].

In children with chronic cholestasis, repeated transcutaneous bedside measurements are a rapid and reliable noninvasive test for characterizing the severity of abnormal oxygenation, and may prove useful also in post liver transplantation monitoring [30]. Although the existence of hypoxemia and hepatopulmonary syndrome has a prognostic value in patients with chronic liver disease [31], controlling the oxygen pressure, is not recommended as a screening tool [9, 10]. Therefore disease in diagnosis and control echocardiography with contrast should be more exact.

In conclusion, garlic can reduce the severity of hepatopulmonary syndrome. Arterial oxygen pressure is increased after treatment with garlic. Further studies are needed to evaluate the garlic therapy for hepatopulmonary syndrome in children.

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RAPID COMMUNICATION

Transfusion-transmitted virus in association with hepatitis A-E viral infections in various forms of liver diseases in India

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Abstract

AIM: To describe the prevalence of transfusion-transmitted virus (TTV) infection in association with hepatitis A-E viral infections in different forms of liver diseases in North India.

METHODS: Sera from a total number of 137 patients, including 37 patients with acute viral hepatitis (AVH), 37 patients with chronic viral hepatitis (CVH), 31 patients with cirrhosis of liver and 32 patients with fulminant hepatic failure (FHF), were analyzed both for TTV-DNA and hepatitis A-E viral markers. Presence of hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis E virus (HEV) infections was detected in different proportions in different groups. Moreover, TTV-DNA was simultaneously tested in 100 healthy blood donors also.

RESULTS: None of the patients had hepatitis A virus (HAV) and hepatitis D virus (HDV) infections. Overall prevalence of TTV-DNA was detected in 27.1% cases with AVH, 18.9% cases with CVH, 48.4% cases with cirrhosis and 9.4% cases with FHF. TTV-DNA simultaneously tested in 100 healthy blood donors showed 27% positivity. On establishing a relation between TTV infection with other hepatitis viral infections, TTV demonstrated co-infection with HBV, HCV and HEV in these disease groups. Correlation of TTV with ALT level in sera did not demonstrate high ALT level in TTV-infected patients, suggesting that TTV does not cause severe liver damage.

CONCLUSION: TTV infection is prevalent both in patients and healthy individuals in India. However, it does not have any significant correlation with other hepatitis viral infections, nor does it produce an evidence of severe liver damage in patients with liver diseases.

INTRODUCTION

In 1997, a novel DNA virus was identified in serum of a Japanese patient (TT) having post-transfusion hepatitis[1]. This virus was designated as transfusion-transmitted virus (TTV). Later studies demonstrated TTV to be a non-enveloped single-stranded DNA virus, whose genome consists of 3739 bp and two large overlapping open reading frames (ORF-1 and ORF-2) encoding 770 and 202 amino acids and several small ORFs (22-105 amino acids)[2]. The virus has little sequence similarity with any known virus and so, it is presently not clear to which family it belongs. It is likely that TTV represents a new family. These are dense and 40-nm diameter particles. Individual genome sequences vary by up to 40%. Comparison of the genomic sequence, encoded proteins and the biophysical characteristics of the virus suggests that TTV is closely related to the Circoviridae. The genome exhibits high diversity and is classified into 6 genotypes[3]. As the prevalence of TTV in populations that require frequent transfusion of blood products is higher than that in healthy blood donors, TTV was supposed a parenterally transmissible virus. However, since TTV has been reported to be excreted in feces[4] also, it was felt that virus might also be transmitted non-parenterally. Using PCR with a new set of primers derived from a highly conserved region of the TTV genome, TTV DNA was detected in 92% of the general population in Japan[5], demonstrating that TTV is indeed a common virus.

Since its discovery, it has become clear that TTV infection is present worldwide among blood donors and is common in patients with liver diseases, including cryptogenic cirrhosis and fulminant hepatic failure. In one study, TTV DNA was detected in 46% and 12% of non-A to G hepatitis patients and healthy blood

donors, respectively[4], and it was initially suggested that TTV was a new hepatitis virus. The association of TTV with cryptogenic chronic liver diseases, post-transfusional hepatitis[1] and acute hepatitis of unknown etiology suggested an etiological role of this agent in the development of both acute and chronic hepatitis. Preliminary data from Japan and the United Kingdom indicated that TTV sequences were detectable in 25%-47% of patients with fulminant or chronic hepatitis of unknown origin, 27%-68% of hemophiliacs, and 1.9%-22% of apparently healthy blood donors[6-10]. Moreover, TTV-DNA titres were found to be 10- to 100-fold greater in liver tissue than in serum. All these findings have been interpreted as demonstrating its hepatotropism. Evidence for potential hepatotropism of TTV was supported by the presence of high TTV-DNA concentration in liver[7] and by a correlation between TTV-DNA titres and aminotransferase levels in post-transfusional non-A-G hepatitis patients[11]. In some studies, TTV-DNA has been found more frequently in patients with liver cirrhosis and hepatocellular carcinoma than in those with chronic hepatitis. However, virus DNA is not integrated in tumor cells, which may suggest that the virus is a passenger rather than a cause of the tumor. And therefore, further studies are required to determine the role of TTV. In fact, the significance of TTV infection in liver disease is, at present, analogous to that of HGV.

The aim of this study was to evaluate the prevalence of TTV-DNA in patients with different liver diseases in north India, and at the same time, find out the possible relation between TTV and other hepatitis viral infections in causation or progression of different liver diseases.

MATERIALS AND METHODS

Patients and blood samples

One hundred and thirty seven patients of both sexes and in adult age group were included in the present study. The patients consisted of 37 patients with acute viral hepatitis (AVH, age range: 21-48 years), 37 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 31 patients with liver cirrhosis (age range: 34-57 years) and 32 patients with fulminant hepatic failure (FHF, age range: 28-46). All these patients attended either Outpatient Department or were admitted to the Liver Unit of All India Institute of Medical Sciences, New Delhi, from June 2001 to February 2004. They were evaluated clinically and biochemically and their sera were tested for hepatitis viral markers. The diagnosis of different types of liver diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere[12]. AVH was diagnosed when patients exhibited overt jaundice and/or increased alanine aminotransferase levels (at least 3 times above the normal value) documented at least twice at a 1-week interval without any history of pre-existing liver disease. None of the patients had a past history of alcohol intake or using any drug. We also could not find any clinical or serological evidence of autoimmune diseases or biliary infection in these patients. The patients with CVH and cirrhosis of liver were diagnosed by histopathological criteria laid down by International Study Group on Chronic Hepatitis[13]. All these CVH patients had persistent elevation of transaminases level (at least twice the upper limit of normal range) for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. Fulminant hepatic failure was diagnosed if the patients developed hepatic encephalopathy within 4 wk of the onset of acute hepatitis as outlined elsewhere[12]. One hundred age- and sex-matched healthy subjects were used as controls.

From each of the above patients, 6-10 mL of venous blood was drawn and aliquoted in plain tubes without anticoagulant. Serum was separated after centrifugation and then stored at -70℃ until further analysis. Repeated freezing and thawing of serum was avoided as far as possible. These sera samples were used to analyze various hepatitis markers, liver function tests and hepatitis C virus (HCV) core protein.

Hepatitis viral markers

Sera were investigated for hepatitis B surface antigen (HBsAg) and IgM antibodies to hepatitis A virus (IgM anti-HAV), hepatitis B core antigen (IgM anti-HBc), hepatitis D virus (IgM anti-HDV) and hepatitis E virus (IgM anti-HEV). Similarly, all these sera were also tested for total antibodies against hepatitis C virus (anti-HCV). The serological analysis was done using enzyme immunoassay kits of high sensitivity and specificity. Kits for HBsAg, IgM anti-HBc and IgM anti-HAV were purchased from Abbot Laboratories, USA. Anti-HCV was tested using highly sensitive third generation ELISA kit from Ortho Diagnostics. This anti-HCV kit used peptides *versus* core, NS3, NS4 and NS5 regions of HCV genome, as antigen to coat the ELISA plate. IgM antibody to hepatitis D virus (HDV) was tested using an enzyme immunoassay kit from Wellcome, UK. Similarly, IgM anti-HEV was tested using third generation ELISA kit from Genelabs and Diagnostics, Biotechnology, Singapore.

Detection of HCV-RNA by RT-PCR

Total RNA was isolated from 100-μL serum or plasma using High Pure Isolation kit from Roche, Germany according to manufacturer's instructions. Five micrograms of the isolated RNA were applied to reverse transcription and nested PCR with primers located in the highly conserved 5' noncoding region (5' NCR) using BIOHCV kit (B&M Labs., Madrid, Spain). The reverse transcription mixture was incubated for 1 min at 85℃, followed by 30 min at 60℃. First PCR was performed in whole content after adding 40 μL of HCV amplification mixture. Thermal cycler was programmed as follows: 85℃ for 30 s, 94℃ for 2 min, followed by 40 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s and then further incubation of samples for 5 min at 72℃. Five microliters of first PCR product were subjected to nested PCR using nested PCR mixture containing second round primer and enzymes. The protocol on thermo cycler was the same as mentioned in first PCR. The PCR product was electrophoresed on 20 g/L agarose containing ethidium bromide and visualized under UV. A positive control provided in the kit was used as control. All positive and negative controls were tested in parallel with test samples throughout the entire procedures,

Table 1 Prevalence of TTV-DNA in different liver diseases

Liver disease	*n*	Presence of TTV-DNA *n* (%)
Acute viral hepatitis	37	10 (27.0)
Chronic viral hepatitis	37	7 (18.9)
Cirrhosis	31	15 (48.4)
Fulminant hepatic failure	32	3 (9.4)
Control	100	27 (27.0)

starting with RNA extraction.

Detection of TTV-DNA

Total DNA was extracted from 200 μL of serum using DNA isolation kit from Roche Diagnostics GmbH, Germany. This isolation method utilizes the ability of nucleic acids to absorb to silica (glass) in the presence of a chaotropic salt. Serum sample was treated with buffer containing proteinase K and silica particles where nucleic acids are bound to silica surface of magnetic particles. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by washing. A low salt buffer is used to elute the DNA. Using 5 μL of the DNA solution as a template, TTV DNA of the open reading frame (ORF-1) sequence, was detected by PCR employing the semi-nested primers reported by Okamoto *et al*[2]. The first-round PCR was carried out for 35 cycles, each cycle consisting of denaturation at 94℃ for 45 s, primer annealing at 60℃ for 45 s and extension at 72℃ for 60 s, followed by an additional extension at 72℃ for 7 min, using the primers NG059 (sense: 5'-ACAGACAGAGGAGAAGGCAAC ATG-3') and NG063 primer (antisense: 5'CTGGCATT TTACCATTTCCAAAGTT-3'). Thereafter, the second-round PCR was carried out using 1 μL of the first-round PCR product, NG061 primer (sense: 5'-GGCAACATG YTRTGGGATAGACTGG-3', where Y=T or C; R=A or G), and NG063 primer for 25 cycles under the same aforementioned conditions. The PCR product (10 μL) was electrophoresed on 20 g/L agarose gel containing ethidium bromide, and observed under ultraviolet light. The product of the first-round PCR was of 286 bp and that of the second-round PCR was of 271 bp.

Diagnosis of viral hepatitis

Liver function tests, including transaminase levels (AST and ALT) in serum, were performed on autoanalyser Hitachi-917 using the established techniques. Similarly, hemogram and coagulation profiles were performed using routine assays established in our laboratory. The diagnosis of different types of viral hepatitis was established as follows: The diagnosis of hepatitis A virus (HAV) infection was confirmed by the presence of IgM anti-HAV in serum. Hepatitis B virus (HBV) infection was established by presence of IgM anti-HBc in sera of AVH and FHF patients and by the persistent HBsAg antigenemia in sera of CVH and cirrhosis cases. Similarly, anti-HCV and IgM anti-HDV in sera samples were used for the diagnosis of HCV and HDV infections, respectively. All anti-HCV sera were also tested for HCV-RNA using nested PCR as described above. However, final diagnosis of HCV was based on anti-HCV antibodies in serum. Active or recent hepatitis E virus (HEV) infection was diagnosed by the presence of IgM anti-HEV in serum. Sera positive for HBsAg but negative for all other viral markers were labeled as HBV-carriers. Absence of all the markers including HBsAg labeled the patients with hepatitis non-ABCDE infection. HCV-RNA in serum was used to confirm active HCV infection.

RESULTS

Analysis of sera for different hepatitis viral markers demonstrated the presence of hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV) infections in these patients. None of the sera analyzed could demonstrate the presence of markers related to hepatitis A virus (HAV) and hepatitis D virus (HDV) infections. HBV infection was detected in 19 of 37 (54.1%) patients with AVH, 30 of 37 (80.1%) with CVH, 18 of 31 (58.1%) with cirrhosis and 13 of 32 (40.6%) with FHF. HCV infection, as indicated by the presence of anti-HCV in serum, was detected in 8 of 37 (21.6%) patients with AVH, 15 of 37 (40.5%) with CVH, 5 of 31 (16.1%) with cirrhosis and 2 of 32 (6.3%) with FHF. All anti-HCV-positive sera were also tested for HCV-RNA which was found positive in 96-98% sera samples. The percentage of HCV infection was based on anti-HCV positivity in these cases. HEV infection was observed in 14 of 37 (37.8%) cases with AVH and 5 of 32 (15.6%) cases with FHF.

Presence of TTV-DNA was detected in 10 of 37 (27.0%) cases with AVH, 7 of 37 (18.9%) cases with CVH, 15 of 31 (48.4%) cases with cirrhosis and 3 of 32 (9.4%) cases with FHF (Table 1). TTV-DNA was also detected in 27 of 100 (27%) healthy blood donors. In order to investigate TTV coinfection with different hepatitis viral infections, we observed TTV-DNA with IgM anti-HBc in 16.2% cases with AVH and none with FHF. TTV-DNA with HBsAg, indicating TTV-HBV co-infection in CVH and cirrhosis, was observed in 13.5% and 32.3% cases, respectively. TTV-HCV coinfection, indicated by simultaneous presence of TTV-DNA and anti HCV, was present in 10.8% case with CVH, 6.5% with cirrhosis and 3.2% cases with FHF. None of AVH patients had TTV-HCV coinfection. TTV-HEV coinfection could be demonstrated in 2.7% cases with AVH only. No other disease group had HEV-TTV coinfection (Table 2).

When TTV-DNA positive cases were analyzed for ALT level in sera, we found that 60% AVH patients had ALT level up to 3 334 nkat/L, 30% up to 6 668 nkat/L and only 2.7% more than 10 002 nkat/L. In CVH, all TTV-positive cases had ALT level less than 3 334 nkat/L. Similarly, in cirrhosis patients, ALT level was found up to 3 334 nkat/L in 93.3% cases and up to 6 668 nkat/L in 6.7% cases. In FHF, all three cases had ALT level more than 10 002 nkat/L. ALT level in serum was used as an index of liver damage in different disease groups (Table 3).

DISCUSSION

To investigate the status of TTV coinfection in relation

Table 2 Presence of TTV-DNA in relation to other viral hepatitis markers in liver diseases

Liver disease	*n*	*n* (%) TTV-DNA with HBsAg/IgM anti-HBc	TTV-DNA with anti-HCV/HCV-RNA	TTV-DNA with IgM anti-HEV
Acute viral hepatits	37	6 (16.2)	0 (NIL)	1 (2.7%)
Chronic viral hepatitis	37	5 (13.5)	4 (10.8)	-
Cirrhosis	31	10 (32.3)	2 (6.5)	-
Fulminant hepatic failure	32	0 (NIL)	1 (3.2)	0 (NIL)

NIL: 0%. - : Not done, as HEV is not present in chronic and cirrhosis cases.

Table 3 Relation between TTV-DNA and ALT levels in different liver disease groups

Liver disease	No. of TTV-DNA + patients	*n* (%)[ALT (nkat/L)] 833.5 - 3334	3335 - 6668	6669 - 10002	>10002
Acute viral hepatitis	10	6 (60)	3 (30)	0 (NIL)	1 (10)
Chronic viral hepatits	7	7 (100)	0 (NIL)	0 (NIL)	0 (NIL)
Cirrhosis	15	14 (93.3)	1 (6.7)	0 (NIL)	0 (NIL)
Fulminant hepatic failure	3	0 (NIL)	0 (NIL)	0 (NIL)	3 (100)

Present value was computed in comparison to total number positive for TTV-DNA. Nil : 0%.

to other hepatitis infections in different liver diseases, we tested TTV-DNA simultaneously with markers related to hepatitis A-E infections. Results are shown in Table 2. Analysis of data showed that TTV-HBV coinfection was detected in all the groups except FHF. Similarly, TTV-HCV coinfection was also recorded in all except AVH group. TTV-HEV coinfection was found in a minor proportion of AVH group. Attempts were also made to study the impact of TTV infection on liver damage both with or without other hepatitis viral infections. For this, serum level of ALT was tested in all the cases. The results showed that TTV infection did not cause severe liver necrosis in these groups of patients. This was indicated by a very moderate elevation in ALT level in the disease groups except FHF, which otherwise always showed a high ALT level.

While reviewing our findings in reference to various other studies, we found that our data are in agreement with several other reports. Earlier reports indicate that a majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT level and do not develop chronic hepatitis, although TTV viremia persists for years[9,19-20]. We found TTV-DNA in 27% normal population with normal ALT level and at the same time no significant increase in ALT in hepatitis A-E, TTV-positive patients, thereby suggesting that TTV alone does not cause much change in ALT level. This raises the possibility that TTV is merely an innocent bystander, both with and without non A-E hepatitis viral infections, and does not add to the damage caused by other hepatotropic viruses. Also, as such it does not appear to be the primary cause of hepatitis, though many more studies are still needed to prove this theory. The results by Vimolket *et al*[21] demonstrated that in non A-E hepatitis cases, mean ALT level was comparable among TTV-positive and negative cases. There was no consistent relationship between ALT and TTV-DNA level among these patients. These results also support our findings.

In conclusion, TTV infection is prevalent both in normal as well as patients populations in India. It shows its presence in all types of liver diseases, though comparable to the normal population and presenting no evidence of increasing liver damage both with or without other hepatitis viral infections. TTV infection has been found to be coinfected with HBV, HCV and HEV infections, but does not significantly increase ALT level in these patients. It appears as if TTV is a benign virus acting as a bystander in the body without causing any damage of the liver.

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RAPID COMMUNICATION

Identification of a new target region on the long arm of chromosome 7 in gastric carcinoma by loss of heterozygosity

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Abstract

AIM: To define the common deleted region on the long arm of haman chromosome 7q linked to primary gastric carcinomas in Chinese by loss of heterozygosity (LOH) and its clinical significance.

METHODS: Nine microsatellite markers distributed over chromosome 7q with an average marker density of 10cM were used to examine 70 primary gastric carcinomas for LOH by PCR amplification. The PCR products were separated by electrophoresis on polyacrylamide gel. Genescan and Genotyper softwares were used to analyze LOH.

RESULTS: LOH with at least one marker on 7q occurred in 34.3% (12/50) of the tumors. Among them, LOH at D7S486 and D7S798 was higher in 24.0% (24/70) and 19.2% (5/26), respectively. By statistical analysis we also observed an obvious genotype-phenotype correlation on 7q ($P < 0.05$). The frequency of LOH at D7S486 in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis ($P = 0.015$).

CONCLUSION: The high incidence of LOH at D7S486 and its correlation with poorer prognosis suggest that there might be putative tumor suppressor genes in this region involved in the tumorigenesis and progression of gastric carcinoma.

Key words: LOH; Chromosome 7; Gastric cancer

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies in the digestive system. Generally, the incidence and mortality vary greatly depending on the region. The highest incidence is found in Japan and China in comparison with other regions of the world. It is one of the leading causes of cancer-related death[1]. Multi-gene and multi-step changes are involved in the occurrence and progression of tumors, including activation of oncogenes and inactivation of tumor suppressor genes (TSG). However, the vital genetic alterations remain uncertain. Previous genetic researches have demonstrated highly frequent amplifications of 1q, 2q, 6q, 7q, 8q, 17q, 20q and deletions of 1p, 1q, 2p, 3p, 4q, 5q, 6p, 7q, 9p, 11p, 11q, 12q, 15q, 17p, 17q, 18q, 19p, 21q in primary gastric cancers[2-5], indicating that these genetic alterations might relate to the occurrence and progression of gastric carcinoma. We used direct G-banding analysis and FISH on de-colored G-banding to study chromosome aberrations in primary gastric carcinoma and detected the deletion of 7q, 3p, 1p and 17p. The frequency of 7q deletion was particularly high[6], suggesting that there might be a potential TSG on the long arm of chromosome 7 involved in the progression of gastric carcinoma. Given the high rate of morbidity and mortality associated with GC, any means of reducing the occurrence of the disease or increasing its early detection is most desirable. In this study we examined the loss of heterozygosity (LOH) in microsatellite locus on human chromosome 7q of primary gastric carcinoma and corresponding non-tumor gastric mucosa tissues in order to narrow the oriented region of related TSG on 7q.

Table 1 Distribution and frequency of LOH at 9 microsatellite loci in primary gastric carcinomas

Microsatellite loci	Location on chromosome	LOH cases	Heterozygous cases	Positive rate of LOH (%)
D7S515	7q22.1	6	53	11.3
D7S486	7q31.1	12	50	24
D7S530	7q32.1	4	50	8
D7S640	7q33	3	63	4.8
D7S684	7q34	4	58	6.9
D7S661	7q35	3	27	11.1
D7S636	7q36.1	3	60	5
D7S798	7q36.2	5	26	19.2
D7S2465	7q36.3	5	52	9.6

MATERIALS AND METHODS

Materials

Primary gastric cancer and its corresponding normal tissue were obtained from 70 patients resected at the Affiliated Cancer Center of Zhongshan University during December 2000 to January 2002. All the patients did not receive any radiotherapy or chemotherapy before operation. The tissues were taken immediately after excision. One part of the samples was preserved at -80℃ for DNA extraction, the others were treated with routine protocol, embedded with paraffin and stained with HE.

Seventy patients (52 men and 18 women) aged 19 - 76 years (mean age 52.9 years) were enrolled in this study. The histological types included 1 papillary adenocarcinoma, 15 tubular adenocarcinomas, 25 poorly-differentiated adenocarcinomas, 2 mucinous adenocarcinomas, 20 signet-ring cell adenocarcinomas, 6 undifferentiated adenocarcinomas and 1 adenosquamous carcinoma, which were diagnosed according to the WHO classification of gastric carcinoma (1990). Based on the UICC TNM classification of gastric carcinoma, the patients were divided into 7 cases of stage Ⅰ (3 of ⅠA and 4 of ⅠB), 13 cases of stage Ⅱ, 24 cases of stage Ⅲ (14 of ⅢA and 10 of ⅢB) and 26 cases of stage Ⅳ.

DNA extraction

Genomic DNA samples of cryo-preserved gastric carcinomas and matched normal gastric mucosa were isolated by phenol chloroform extraction using standard protocols. DNA purification and concentration were detected by gel electrophoresis.

Selection of microsatellite markers and PCR amplification

A total of 9 microsatellite markers distributed over the whole chromosome 7q were selected from Genethon human genetic linkage map: D7S515, D7S486, D7S530, D7S640, D7S684, D7S661, D7S636, D7S798 and D7S2465. The frequencies of heterozygosity of the markers were over 75% and the average marker density was about 10cM. Synthesized microsatellite primers were respectively labeled with three different color fluorescent dyes: FAM, NED and HEX (purchased from PE Company). DNA samples were divided into 2 groups according to molecular size of the products and color of the fluorescent dyes. Primers (including 4 to 5 pairs) of each group were mixed in a tube for amplification. Each multiplex PCR reaction volume (5 μL in total) contained 0.5 μL 10×buffer, 0.2 μL 10 mmol/L dNTP, 0.3 μL 25 mmol/L $MgCl_2$, 0.05 μL 10 pmol/L primers, 0.2 U hot star Taq™ DNA polymerase (Gene Company, USA) and 45 ng genomic DNA. PCR amplifications were carried out in Gene Amp PCR system 9700 thermal cycler (Perkin-Elmer Co, Norwalk, USA). The reactive conditions were as follows: initial denaturation at 95℃ for 12 min followed by 10 cycles at 94℃ for 30 s, at 63℃ for 60 s (decreasing by 0.5℃ per cycle), at 72℃ for 90 s, and 26 cycles at 94℃ for 30 s, at 58℃ for 60 s, at 72℃ for 90 s and a final extension at 72℃ for 15 min.

Genomic scanning and analysis of microsatellite instability

PCR products (0.5μL) were diluted with 1.0 μL electrophoresis loading buffer containing 70% formamide, 3.75 mmol/L EDTA, 7.5 mg/ml blue dextran, 1.2 nmol/L Genescan 350. Mixture (1.0 μL) was loaded in urea-containing denaturing Sequagel Tm polyacrylamide gel (Gene Company, USA), and subjected to electrophoresis on a 377 DNA sequencer (ABI PRISM, PE Applied Biosystems). The automatically collected data were analyzed with Genescan Version 3.0 and Genotyper Version 2.0 software. Genotype for a given locus with two peaks of PCR fragments was defined as heterozygosity, and genotype with a single peak was regarded to be homozygosity. Only patients heterozygous for a given locus were regarded to be informative. Comparison of the ratios between tumors (T) and their controls (N) was made using the following formulas for calculations:

$(T_1/T_2)/(N_1/N_2)$ (1)

$(T_2/T_1)/(N_2/N_1)$ (2)

where T_1 and N_1 are the peak height of the smaller allele, while T_2 and N_2 are the peak height of the larger allele. Formula (1) was used to calculate the ratio of the smaller allele while formula (2) was used to calculate the ratio of the larger allele. For ratios greater than 1, the reciprocal of the ratio was calculated to give a value between 0.00 and 1.00. A value <0.67 was assigned as indicative of LOH[10-12].

Statistical analysis

The data of two-sample ratio were evaluated by chi-square test and Fisher's exact test. $P<0.05$ was considered statistically significant.

RESULTS

Distribution and frequency of LOH in primary gastric carcinoma

Genomic DNA was extracted from 70 primary gastric carcinomas and paired normal gastric mucosa tissues, 9 microsatellite fragments were amplified. Subsequent genotype analysis of the reliable amplification fragments was performed. LOH at all 9 loci was found in 70 primary gastric carcinoma cases (Table 1). The highest frequency of LOH was 24.0% at D7S486 and 19.2% at D7S798, respectively. LOH was detected in 24/70 (34.3%) of

Table 2 Correlation between frequencies and clinicopathology of LOH in primary gastric carcinomas

Clinicopathological variables		LOH frequency of chromosome 7 (%)	χ^2	*P* value	LOH frequency of D7S486 (%)	χ^2	*P* value
Gender	Male	17/52 (32.7)	0.22	0.636	7/35 (20)	1	0.317
	Female	7/18 (38.9)			5/15 (33.3)		
Age (yr)	>53	14/37 (37.8)	0.43	0.51	7/26 (26.9)	0.25	0.618
	≤53	10/33 (30.3)			5/24 (20.8)		
Clinical stage	I-II	3/20 (15)	6.16	0.046	1/13 (7.7)	2.63	0.268
	III	8/24 (33.3)			5/18 (27.8)		
	IV	13/26 (50)			6/19 (31.6)		
T stage	T1-2	3/13 (23.1)	0.88	0.349	1/8 (12.5)	0.68	0.411
	T3-4	21/57 (36.8)			11/42 (26.2)		
Lymph node metastasis	No	4/19 (21.1)	2	0.158	0/13 (0)		0.015
	Yes	20/51 (39.2)			12/37 (32.4)		
Distance metastasis	No	20/60 (33.3)	0.17	0.683	9/42 (21.4)		0.379
	Yes	4/10 (40)			3/8 (37.5)		
Histopathological type	Tubular adenocarcinoma	5/15 (33.3)	2.79	0.425	2/9 (22.2)	0.12	0.989
	Poorly- differentiated carcinoma	9/25 (36)			6/22 (27.3)		
	Signet-cell adenocarcinoma	6/19 (31.6)			3/12 (25)		
	Undifferentiated adenocarcinoma	4/6 (66.7)			1/4 (25)		

primary gastric carcinomas, 15/24 cases (62.5%) showed LOH at only one locus, 9/24 (37.5%) cases had LOH at two loci, and LOH occurred at more than three loci in 5/24 cases (25.0%).

Correlations between LOH and clinicopathology

Relations between LOH frequency and clinicopathology in 70 primary gastric carcinomas are shown in Table 2. There were statistical correlations between LOH frequencies at 7q and clinical stages. The frequency increased with poorer clinical stages ($P=0.046$). LOH at all 9 loci was detected in 13/26 (50.0%) cases with clinical stage Ⅳ, of which 6 cases (46.1%) were found to have LOH at D7S486. Five cases with LOH at more than 3 loci were all in stage Ⅳ. The frequency of LOH at D7S486 was related with lymph node metastasis. It was significantly higher in cases with lymph node metastasis than in those without metastasis ($P=0.015$).

However, frequencies of LOH showed no statistically differences in tubular adenocarcinomas, poorly-differentiated adenocarcinomas, signet-cell carcinomas and undifferentiated adenocarcinomas. Four cases showed LOH in 6 undifferentiated adenocarcinomas.

DISCUSSION

In our previous study, chromosome aberrations and their roles in the genesis and development of primary gastric cancer were investigated using direct G-banding analysis and FISH[7]. The deletion of chromosome 7q is the most consistent aberration, and 7q31-qter is the commonly lost segment[7-9]. LOH of this region is a very common occurrence in many kinds of human malignancies including cancers of breast[10], prostate[11], colon[12] and ovary[13], as well as primary squamous cell carcinoma of the head and neck[12]. Taken together, a critical TSG probably exists in this region with activation in a broad range of tissues. Some putative TSGs in this region such as ST7, Caveolin-1, ING3, and PPP1R3 have been reported[7,9,14,15]. However, no further researches provide reliable evidence for the correlation between these candidate genes and primary gastric carcinomas.

Tumor occurrence and progression involve multi-genes and multi-steps. Different genetic alterations participate in tumor occurrence and progression, and genetic alteration plays a vital role in different tumors. Several chromosomal amplifications and deletions have been reported in primary gastric carcinomas[2-5]. Kuniyasu *et al*[16] have reported LOH at 5 microsatellite markers on 7q in 32% (26/82) of 98 gastric carcinomas. D7S95 on 7q31-35 is the most frequent change locus. Similar results were also reported by Nishizuka *et al*[17]. Our findings are consistent with these previous studies. In our study, the total LOH at 7q was 34.3% in gastric carcinomas, and the frequency of LOH at D7S486 reached 24.0%. The higher frequencies of LOH at D7S486 and D7S798 than at the other 7 loci indicate the presence of tumor suppressor genes in these regions, particularly near D7S486.

Different results about correlations between LOH and clinical factors of chromosome 7q have been reported in various studies. Kuniyasu *et al*[16] found that LOH at D7S95 on 7q31-35 is much higher in stage Ⅳ gastric carcinomas and that patients with LOH at D7S95 show celiac metastasis compared with those without LOH ($P<0.05$). Moreover, patients of stage Ⅲ-Ⅳ with LOH at D7S95 survive shorter than those without LOH ($P<0.05$). Thus, LOH at D7S95 is likely involved in gastric carcinoma progression and prognosis. In our 70 gastric carcinomas, the frequency of LOH at any locus on 7q increased obviously with the rising of clinical stage ($P=0.046$), and reached 50.0% (13/26) in patients with clinical stage Ⅳ. Moreover, 5 cases with LOH at more than 3 loci were all in stage Ⅳ. The frequency of LOH at D7S486 in patients with lymph node metastasis was obviously higher than that in those without lymph node metastasis ($P=0.015$). There was no significant correlation between LOH and histological types. This lack of correlation may be related to the small number of undifferentiated tumors. Our results suggest

that one or more tumor suppressor genes associated with gastric carcinomas might situate on chromosome 7q and D7S486. Loss of restraining effects on tumor proliferation, infiltration and metastasis of these candidate genes might promote gastric carcinoma progression.

The region around the marker D7S486 may contain a fragile site. In fact, a 7q31.2 fragile site (FRA7G) of 300 kb is located between markers D7S486 and D7S522[18]. FRA7G is a common aphidicolin-inducible fragile site at 7q31.2, showing LOH in human malignancies. Common fragile sites are specific regions in mammalian chromosomes that are prone to breakage and rearrangements. This genetic instability can lead to disease manifestations and may play a role in oncogenesis[19]. The present study delineated a breakpoint of putative TSG near the marker D7S486. Tatareli *et al*[20] investigated the structure of FRA7G spanning the region between marker D7S486 and Met H and have identified a gene encoding a 421-amino-acid protein with three LIM domains with 89% identity to murine Testin. These findings suggest that TESTIN may represent a candidate tumor suppressor gene at 7q31.2.

The genetic intervals of microsatellite markers in our study were relatively wide (10cM). Additional studies are needed to narrow these regions on D7S486 and identify potential tumor suppressor genes.

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RAPID COMMUNICATION

Perioperative artificial nutrition in malnourished gastrointestinal cancer patients

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Abstract

AIM: To investigate the potential role of perioperative nutrition in reducing complications and mortality in malnourished gastrointestinal cancer patients.

METHODS: Four hundred and sixty-eight elective moderately or severely malnourished surgical patients with gastric or colorectal cancers defined by the subjective global assessment (SGA) were randomly assigned to 7 d preoperative and 7 d postoperative parenteral or enteral nutrition *vs* a simple control group. The nutrition regimen included 24.6±5.2 kcal /kg per d non-protein and 0.23±0.04 g nitrogen /kg per d. Control patients did not receive preoperative nutrition but received 600±100 kcal non-protein plus or not plus 62 ± 16 g crystalline amino acids postoperatively.

RESULTS: Complications occurred in 18.3% of the patients receiving nutrition and in 33.5% of the control patients ($P = 0.012$). Fourteen patients died in the control group and 5 in those receiving nutrition. There were significant differences in the mortality between the two groups (2.1% *vs* 6.0%, $P = 0.003$). The total length of hospitalization and postoperative stay of control patients were significantly longer (29 *vs* 22 d, $P = 0.014$) than those of the studied patients (23 *vs* 12 d, $P = 0.000$).

CONCLUSION: Perioperative nutrition support is beneficial for moderately or severely malnourished gastrointestinal cancer patients and can reduce surgical complications and mortality.

Key words: Malnutrition; Perioperative nutrition; Parenteral nutrition; Enteral nutrition; Mortality; Complications

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INTRODUCTION

Malnutrition is common in gastrointestinal cancer patients and the causes are often complex and multi-factorial. Although it is widely accepted that malnutrition adversely affects the postoperative outcome of patients, there is little evidence that perioperative nutrition support can reduce surgical risk in malnourished cancer patients. Early retrospective studies suggested that perioperative nutrition support may effectively reduce postoperative complications[1,2]. Subsequent prospective, randomized clinical trials (RCTs) demonstrated that the benefits of perioperative nutrition support are limited to severely malnourished patients undergoing major surgery[3,4]. Most RCTs of perioperative nutritional support in patients with gastrointestinal cancer have shown that the degree of malnutrition varies considerably, ranging from no weight loss to weight loss exceeding 10 % and the results obtained by these studies are different in malnourished and non-malnourished patients[1-4]. Unfortunately, studies in really malnourished gastrointestinal cancer patients are not available. Meanwhile, parenteral and enteral nutrition is till controversial in perioperative malnourished gastrointestinal cancer patients[5].

The aim of this study was to evaluate the efficacy of optimal perioperative nutrition support in reducing complications and mortality in malnourished gastrointestinal cancer patients.

MATERIALS AND METHODS

This prospective study was carried out from May 2002 to July 2004 at the General Surgical Department of Zhongshan Hospital. All patients who underwent surgery for gastrointestinal (stomach, colon and rectum) malignancies were eligible for inclusion. Patients were excluded if they were admitted for emergency surgery. Within 48 h of admission, patients underwent nutritional assessment by the subjective global assessment. (SGA) performed with a standardized questionnaire including the patient's history (weight loss, changes in dietary intake, gastrointestinal symptoms, and functional capacity), physical examination (muscle, subcutaneous fat, sacral

Table 1 Preoperative characteristics of the patients

	Study group ($n=235$)	Control group ($n=233$)
Mean age (yr)	57.3	56.5
Male/ female	162/95	166/89
Body weight (kg)	59.4±15.2	61.1±14.9
SGA score		
B	147	153
C	88	80
Gastric carcinoma	124	129
Colon carcinoma	65	61
Rectum carcinoma	46	43

SGA: subjective global assessment; B: moderately- malnourished; C: severely-malnourished.

and ankle edema, ascites) and the clinician's overall judgment of the patient's status (normal, moderately or severely malnourished). On the basis of these data, the patients were classified as well-nourished, moderately- or severely-malnourished. A total of 512 moderately- and severely-malnourished gastrointestinal cancer patients were included in this study. The Ethical Committee of the Institution approved this clinical study.

After stratified for age, sex and tumor localization (gastric, colorectal), patients were randomly divided into study group ($n=257$, 95 women, 162 men) and control group ($n=255$, 89 women, 166 men). The mean ages were 57.3 years (range 21 - 84 years) and 56.5 years (range 24 - 86 years) in the study group and control group, respectively.

Perioperative nutrition was administered in the study group by parenteral or enteral route or a combination of the two based on a clinical assessment of intestinal function. Patients due to permanent or temporary intestinal failure were given parenteral nutrition (PN). If the clinician felt that the patients had a functioning gastrointestinal tract, they received enteral nutrition (EN).

Most patients (68%) received PN support during the preoperative and postoperative periods. The PN regimen consisted of 25 kcal/kg per d non-protein and 0.25 g nitrogen /kg per d. The non-protein calorie source included glucose and fat, accounting for 60% and 40% respectively of the energy intake. The protein source was supplied by crystalline amino acid solutions. Electrolytes, vitamins, and trace elements were administered according to the current recommendations. PN mixture was delivered through a central venous catheter or peripheral veins using an "all in one" bag. In addition, the patients receiving preoperative PN had free access to food they preferred.

EN was given to 75 patients (32%) in whom GI function was adequate through a fine bore silicone feeding tube. Where appropriate, EN was alternatively administered via a nasogastric tube or a feeding jejunostomy catheter. Similar target intake of non-protein (25 kcal/kg per d) and protein (0.25 g nitrogen/kg per d) was provided using commercially available enteral formulas. The initial rate of delivery was 40-60 mL/h, increasing stepwise to full intake for 48 h according to patient tolerance. Foods were usually given as a continuous infusion using a volumetric pump for 24 h. Nutritional support was started 8-10 d before surgery and continued for more than a week after surgery.

Patients in the control group were given a standard hospital oral diet before surgery and a hypocaloric parenteral solution (600 kcal non-protein and 60 g amino acid) in the postoperative period until gastrointestinal function recovered completely.

After surgery, patients were monitored daily for postoperative complications including septicemia, intra-abdominal abscess, wound infection, wound dehiscence, fistula formation, urinary tract infection, pneumonia, respiratory insufficiency and phlebitis. Rigid objective criteria were established defining each complication to avoid subjective bias. A diagnosis of septicemia was based on a positive blood culture, hypotension and hypoperfusion. An intra-abdominal abscess was defined as an intra-abdominal purulent collection requiring operative drainage. Fistulae were radiographically documented. A diagnosis of urinary tract infection required a quantitative culture of greater than 100 000 organisms. Pneumonia was documented by an abnormal chest x-ray, positive sputum culture, and treatment with antibiotics. The presence of a wound infection was defined by culture and operative or spontaneous drainage of purulent materials. A wound dehiscence required operative re-closure of the wound. The occurrence and cause of death during hospitalization and the length of hospitalization were recorded.

Statistical analysis

Data were analyzed using standard statistical software (SPSS 10.0). For normally distributed data, a paired Student's t test was used for statistical analysis. $P \leqslant 0.05$ was considered statistically significant. Data were expressed as mean $\pm$ SE.

RESULTS

A total of 512 malnourished patients gave their consent to participate in the study and 16 declined. Of the 512 patients, 28 were not randomized after surgery because of un-resectability. Four hundred and sixty-eight patients were assigned at random to study group ($n=235$, 87 women, 148 men) and control group ($n=233$, 90 women, 143 men). Patient demographics and preoperative parameters of the two groups are presented in Table 1. There were no significant differences in mean age, sex distribution and nutrition status between the two groups. Operative data are listed in Table 2. The mean length of the surgical procedure and the volume of intraoperative blood transfusions were similar in two groups. The volume of postoperative blood transfusions was larger in control group. However, none of these differences was statistically significant. The number of patients requiring albumin infusions and the volume of infused albumin were comparable between the groups.

The number of postoperative complications per study group is shown in Table 3. Forty-three complications occurred in 31 patients in the study group, and five patients died due to major complications. Seventy-eight complications occurred in 64 patients of the control group, and 14 patients died. There were significant

Table 2 Operative data of the patients (mean ± SE)

	Study group (*n* = 235)	Control group (*n* = 233)
Mean operating time (min)	210±84	196±102
Operative blood loss (mL)	540±150	525±120
Mean blood transfused (mL)		
Intraoperative	420±80	400±100
Postoperative	200±60	280±120
Patients with albumin infusion (*n*)	156	173
Mean albumin infusion (g)	45±22	55±30
Kind of operation		
Partial gastrectomy	82	90
Total gastrectomy	42	39
Colon resection	65	61
Rectum resection	46	43

Table 3 Postoperative complications in two groups

	Study group (*n* = 235)	Control group (*n* = 233)
Pneumonia	12	23
Urinary tract infection	6	10
Wound infection	11	20
Septicemia	2	5
Intra-abdominal abscess	4	7
Wound dehiscence	3	4
Fistula	2	4
Respiratory insufficiency	2	3
Phlebitis	1	2
Total	43 in 31 patients	78 in 64 patients

differences in the mortality and complications between the two groups (2.1% *vs* 6.0%, $P = 0.003$ for mortality; 18.3% *vs* 33.5%, $P = 0.012$ for complications). In both groups, the most frequent complication was infection related to muscle weakness and/or prolonged immobilization (respiratory insufficiency, phlebitis).

Table 4 summarizes the incidence of septic complications between PN and EN groups. No significant differences were found in the incidence of septic complications between the two groups of nonrandomized patients. In addition, there was no significant difference in the number of septic complications between the two groups.

The total perioperative and postoperative median length of hospitalization was 22 *vs* 29 d in the study group and 12 *vs* 23 d in the control group, respectively. The total length of hospitalization and postoperative stay of the control patients were significantly longer than those of the study patients ($P = 0.014$, $P = 0.000$).

Table 4 Incidence of septic complications in PN or EN group

	PN group (*n* = 160)	EN group (*n* = 75)	*P*
Incidence of septic complications	25 -15.60%	10 -13.30%	0.36
Mean No. of complications per patient (±SE)	0.22±0.03	0.20±0.04	0.19
Mean No. of complications per infected patient (±SE)	1.33±0.05	1.26±0.06	0.22

PN: parenteral nutrition; EN: enteral nutrition.

DISCUSSION

Malnutrition in hospitalized patients is a critical issue associated with a significant increase in morbidity and mortality. Recent surveys have demonstrated that 30 - 50% of hospitalized patients have a certain degree of malnutrition[6,7]. Malnutrition is closely associated with increased morbidity and mortality after major gastrointestinal surgery. Perioperative nutrition support can restore many of biochemical and immunologic abnormalities in malnourished or normal state. However, it is difficult to demonstrate that perioperative nutrition support can significantly reduce surgical complications except in the most severely malnourished patients[8]. Early retrospective studies from 1970s to 1980s suggested that perioperative nutrition support can reduce surgical complications. Subsequent prospective, randomized trials demonstrated that the benefits of perioperative nutrition support are limited to severely malnourished patients undergoing major surgery[3,4]. Most RCTs of perioperative nutritional support in patients with gastrointestinal cancer have shown that the degree of malnutrition varies considerably, ranging from no weight loss to weight loss exceeding 10%, and that the complication rate decreases from 56% in the control arm to 34% in the TPN arm, with no deaths in the latter group[4,9]. Because nutrition support can only ameliorate but not reverse the catabolic response to trauma, it may effectively improve nutritional state and reduce postoperative complications when started preoperatively[10].

This study represented a large randomized clinical trial to explore the role of perioperative nutrition support in moderately- and severely-malnourished gastrointestinal cancer patients. In these patients nutritional support was started 8 ± 10 d before surgery and continued for more than a week after surgery. Nutrition was given by parenteral or enteral routes or a combination of these two. Postoperative complications were defined by rigid objective criteria to avoid subjective bias. This prospective study demonstrated that adequate perioperative nutritional support could effectively reduce the incidence of postoperative complications in moderately- and severely-malnourished gastrointestinal cancer patients. In perioperative nutrition support patients, there was a two-fold reduction in complications ($P = 0.012$) and a three-fold reduction in death ($P = 0.003$). The most dramatic decrease was noted in major septic complications (14.9% *vs* 27.9%, $P = 0.011$) such as pneumonia and wound infection. In addition, the mortality was statistically lower in artificial nutrition support group than in the control group (2.1% *vs* 6.0%, $P = 0.003$). As a consequence of the lower infection rate, the length of hospital stay of the study group was shorter. These results indicate that malnutrition has a negative impact on postoperative outcomes, which may be efficiently controlled by a perioperative nutrition support

that is adequate in quality, quantity, and duration.

Perioperative nutritional support can be administered by PN or EN or their combination. PN has the advantage of easy administration and essentially immediate provision of optimal nitrogen and caloric requirements once the central venous access is established. A major concern with PN in hospitalized patients is the increased risk of septic complications related to immune dysfunction after PN. Unlike PN, EN is not associated with increased infectious complications. In fact, enteral feeding can maintain structural and functional integrity of the gastrointestinal tract and reduce septic complications in critically ill patients. The major disadvantages of EN support are the time delay when attempting to provide complete nutrition by the enteral route and the inability of patients with postoperative abdominal complications to tolerate enteral feeding. Early postoperative EN has fewer septic complications compared to early postoperative PN [11, 12]. Unfortunately, studies in really malnourished cancer patients are not available. The present prospective study demonstrated that there was no statistically significant difference in the incidence of septic complications between PN and EN (15.6% *vs* 13.3%, $P = 0.36$). In addition, there was no significant difference in the number of septic complications per patient or complications per infected patient between PN and EN between the two groups. The results of our study are different from other prospective randomized trials[13-15]. There are several possible explanations for the discrepancies. First, patients are quite different in terms of age, physiologic and nutritional status. Second, the relative protein and energy intake in previous studies are often not comparable, usually being much higher in patients receiving PN than in those receiving EN[16,17]. Excessive energy intake may result in hyperglycaemia and lead to increased septic complications and mortality[18,19]. In the present study, the prescribed target intake for both PN and EN patients was the same (25 kcal /kg per d and 0.25 g N/kg per d). Third, in previous studies, all patients were randomized to receive either PN or EN, with no consideration given to the issue of gut function. Therefore some patients able to tolerate EN were given PN. In the present study, the route of feeding was dictated by an assessment of gut function, ensuring EN was administered only to patients with adequate intestinal function. The results of this study are in accord with the recent studies[20, 21].

In conclusion, perioperative nutrition support can decrease the incidence of postoperative complications in moderately- and severely-malnourished gastrointestinal cancer patients. In addition, it is effective in reducing mortality. Both parenteral support and enteral nutrition support, or their combination can be used in the management of malnourished patients undergoing gastrointestinal surgery.

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RAPID COMMUNICATION

Influence of acid and bile acid on ERK activity, PPARγ expression and cell proliferation in normal human esophageal epithelial cells

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Abstract

AIM: To observe the effects of acid and bile acid exposure on cell proliferation and the expression of extracellular signal-regulated protein kinase (ERK) and peroxisome proliferator-activated receptor γ (PPARγ) in normal human esophageal epithelial cells *in vitro*.

METHODS: *In vitro* cultured normal human esophageal epithelial cells were exposed to acidic media (pH 4.0-6.5), media containing different bile acid (250 μmol/L), media containing acid and bile acid, respectively. Cell proliferation was assessed using MTT and flow cytometry. The expressions of phosphorylated $ERK_{1/2}$ and PPARγ protein were determined by the immunoblotting technique.

RESULTS: Acid-exposed (3 min) esophageal cells exhibited a significant increase in proliferation ratio, S phase of the cell cycle ($P < 0.05$) and the level of phosphorylated $ERK_{1/2}$ protein. When the acid-exposure period exceeded 6 min, we observed a decrease in proliferation ratio and S phase of the cell cycle, with an increased apoptosis ratio ($P < 0.05$). Bile acid exposure (3-12 min) also produced an increase in proliferation ratio, S phase of the cell cycle ($P < 0.05$) and phosphorylated $ERK_{1/2}$ expression. On the contrary, deoxycholic acid (DCA) exposure (> 20 min) decreased proliferation ratio. Compared with bile acid exposure (pH 7.4), bile acid exposure (pH 6.5, 4) significantly decreased proliferation ratio ($P < 0.05$). There was no expression of PPARγ in normal human esophageal epithelial cells.

CONCLUSION: The rapid stimuli of acid or bile acid increase proliferation in normal human esophageal epithelial cells by activating the ERK pathway.

Key words: Acid; Bile acid; Esophageal epithelial cells; Cell proliferation; Extracellular signal-regulated protein kinase

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INTRODUCTION

The incidence of esophageal adenocarcinoma has been rapidly increasing in western countries. With the improvement of investigation devices, reflux esophagitis and Barrett's esophagus (BE) have also been reported in more and more cases in China. In these diseases, duodenogastroesophageal reflux has been established as a strong risk factor[1]. The main components of refluxates are acid and bile acid, whose potential contributions to the esophageal diseases have widely been discussed. However, the effects of acid and bile acid on esophageal cell proliferation and the related signal transduction mechanisms remain unknown. The mitogen-activated protein kinase (MAPK) family, also known as extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 pathways, transmit extracellular growth-regulating signals to effector genes in the nucleus, the activation of the ERK pathway is usually associated with pro-proliferative and antiapoptotic effect[2]. Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily, has recently been shown to be implicated in the development of many digestive tumors[3], with the potential role in the downstream of ERK pathway[4].

In the present study, we examined the effects of acid and bile acid on cell proliferation in normal human esophageal epithelial cells *in vitro*, in association with ERK signaling pathway and PPARγ expression.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibody raised against human cytokeratin 14 (CK14) was purchased from Changdao Biotech Company, Shanghai. Polyclonal antibodies raised against human ERK$_{1/2}$ and phospho-ERK$_{1/2}$ were purchased from Cell Signaling Technology, USA. Monoclonal antibody raised against human PPARγ was purchased from Santa Cruz, USA. Keratinocyte serum-free media (K-SFM) supplemented with bovine pituitary extract (BPE) and recombinant epithelial growth factor (rEGF) were from Gibco, USA. Hydrochloric acid (1 mol/L) was from Sanpu Pure Chemical Industries, Xi'an. Sodium glycocholate (GC), sodium glycodeoxycholate (GDC), sodium glycochenodeoxycholate (GCDC), sodium taurocholate (TC), sodium taurodeoxycholate (TDC), sodium taurochenodeoxycholate (TCDC), and cholic acid (CA), deoxycholic acid (DCA) were purchased from Sigma, USA, and dissolved in K-SFM to 250 μmol/L. Histostain kit was purchased from Zhongshan Golden Bridge Biotech Company, Beijing. RIPA cell lysis kit was purchased from Shenergy Biocolor BioScience Technology Company, Shanghai. BCA protein assay kit and ECL kit were purchased from PIERCE, USA.

Cell culture

Normal esophageal mucosa samples were obtained from surgically resected esophagus with esophageal carcinoma, then confirmed by an experienced pathologist to contain neither macroscopic tumor tissue nor histologically detectable metaplastic cells or cancer cells. Samples were acquired with the signed informed consent from the patient. Samples were collected aseptically, stored in sterile K-SFM at 4℃, and processed within 4 h. Primary culture of normal esophageal epithelial cells was undertaken according to the method described elsewhere[5]. The collected cells were suspended in K-SFM supplemented with BPE and rEGF, and seeded into 75-cm uncoated plastic culture flask in humidified air containing 50 mL/L CO_2 at 37℃. After the initial subculture, an aliquot of cells was grown on coverslips for immunocytochemical stain with CK14 antibody.

Experimental groups

Equally seeded epithelial cells were cultured in K-SFM without BPE and rEGF for 48 h, then divided into 3 groups, acid exposure group: cells were exposed to the acidified medium (pH 4.0-6.5) for 3-60 min; bile acid exposure group: cells were exposed respectively to the medium containing different bile acid (250 μmol/L) for 3-60 min; and mixed exposure group: cells were exposed to the medium containing different bile acid (250 μmol/L) with different pH (4.0 and 6.5) for 3 min. Cells of control group were cultivated in normal media (pH 7.3).

Cell proliferation and cell cycle determination

For cell proliferation assay, 5×10^3 cells/well were plated in 96-well plates. Using MTT assay, changes in cell number were measured 24 h after different exposures. Then 10 μL MTT solution (5 mg/mL) was directly added to the cell cultures. After 4 h, media were removed and cells were lysed with 100 μL of dimethyl sulfoxide. Absorbance at 490 nm was read and data were presented as ratio to control (proliferation ratio).

For cell cycle determination, cells were seeded at 5×10^5/well in 6-well dishes. After 24 h of different exposures, cells were trypsinized and washed in PBS. A pellet of 1×10^6 cells was fixed in 750 mL/L ice-cold ethanol and stored at 4℃ until analyzed. Before flow cytometric analysis, cells were stained with 50 μg/mL RNase and 50 μg/mL propidium iodide. Cells in the G1, S, and G2 phase of the cell cycle and cell apoptotic percentage were determined using flow cytometer (FACSCalibur, Becton Dickinson, USA).

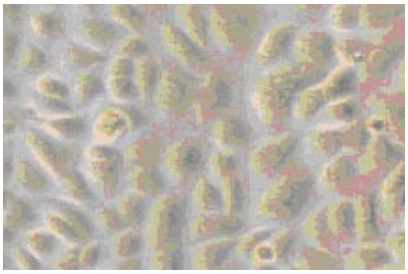

Figure 1 A phase-contrast micrograph of normal human esophageal epithelial cells (× 250).

Immunoblot analysis

After exposure to acid or bile acid for 3 min, cells were immediately treated with cold RIPA cell lysis buffer, followed by centrifugation at 10 000 *g* for 20 min at 4℃ to remove the cell debris. Total protein of the supernatant was measured using a BCA protein assay kit. Protein electrophoresis was performed on a 100 g/L SDS- polyacrylamide gel with 50 μg protein added to each lane. The proteins were then transferred onto a PVDF membrane. The membrane was incubated overnight at 4℃ with the various primary PPARγ (1:100), ERK$_{1/2}$ and anti-phospho ERK$_{1/2}$ (1:1000) antibodies, and then incubated with corresponding secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The protein bands were visualized by ECL and exposed to X-ray film. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System.

Statistical analysis

All experiments were performed in triplicate. The data were expressed as mean ± SD. Statistical analysis between two groups was carried out using Student's *t*-test, for comparison of three or more groups using ANOVA test. $P < 0.05$ was considered statistically significant.

RESULTS

Growth characteristics of normal human esophageal epithelial cells

Epithelial monolayer cultures were successfully established with the appearance of cobblestones (Figure 1). As a cytoskeleton protein of esophageal epithelial cells, CK14 was expressed in all secondary cultures using immunocytochemical stain.

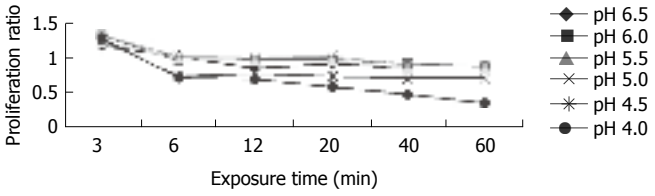

Figure 2 Effect of acid exposure on cell proliferation.

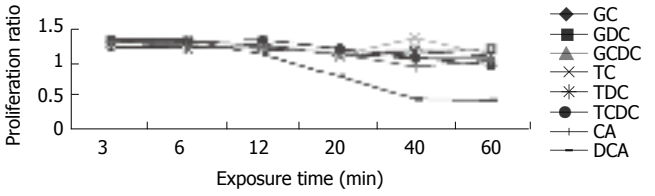

Figure 3 Effect of bile acid exposure on cell proliferation.

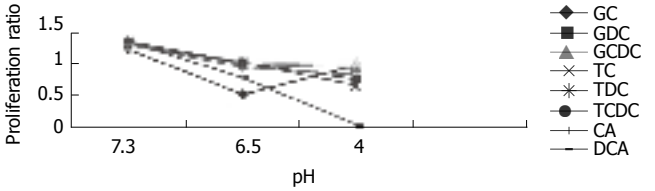

Figure 4 Changes in proliferation of cells exposed to bile acid with different pH for 3 min.

Measurement of cell proliferation and cell cycle

For cells exposed to acid (Figure 2), proliferation ratios were above 1 at 3 min, then decreased gradually to below 1 with prolongation of exposure time, especially when pH < 5.0. For cells exposed to bile acid (Figure 3), proliferation ratios of GC, GDC, GCDC, TC, TDC, TCDC, and CA groups were all above 1 or near 1 with the exposure time of 3-60 min. For DCA group, proliferation ratios were above 1 at 3-12 min, then decreased to below 1 with prolongation of exposure time. For mixed exposure group (Figure 4), proliferation ratios of cells exposed to bile acid with pH 6.5 or 4 for 3 min were markedly less than that of bile acid group (pH 7.4) (P <0.05), for most groups the proliferation ratios dropped to the lowest at pH 4, yet for GC the lowest proliferation ratio was seen at pH 6.5.

The results of cell cycle determination are summarized in Table 1. Compared with the control (pH 7.3), a short-period exposure to acid (3 min) produced an obvious increase of cells in S phase (P < 0.05), and a similar apoptosis percentage. In contrast, longer exposure to acid (6, 12 min) significantly decreased the cell proportion in S phase accompanied by an increased apoptosis percentage (P <0.05). Similarly, a brief exposure to bile acid (3-12 min) also produced an increase of cells in S phase (P<0.05), and a similar apoptosis percentage.

Expression of ERK1/2 and PPARγ

The expression of phospho-ERK1/2 was significantly increased in cells exposed to pH 4.0, 5.0 and 6.0 for 3 min (Figure 5) compared to control (pH 7.3) (P < 0.05). ERK

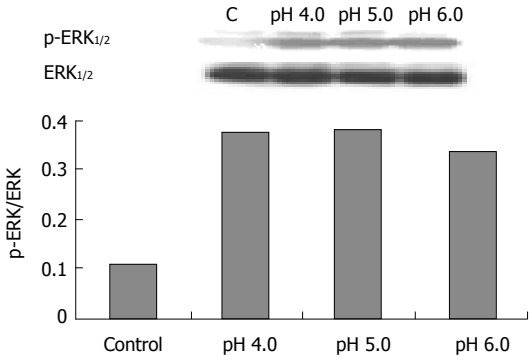

Figure 5 Effect of acid exposure (3 min) on ERK.

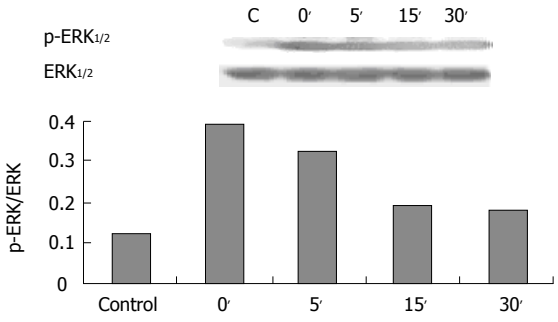

Figure 6 Expression of ERK in 30 min after acid exposure (pH 4.0, 3 min).

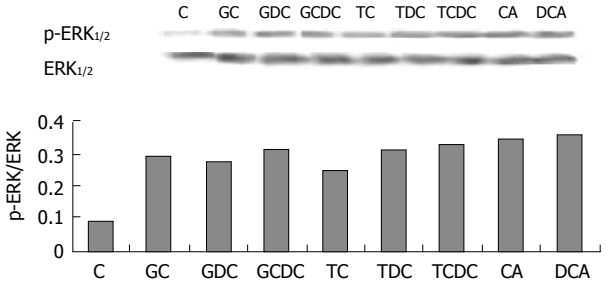

Figure 7 Effect of bile acid exposure (3 min) on ERK.

activity increased immediately after acid exposure (pH 4, 3 min), approximate to the level of control after 15 min (Figure 6). There were also increased expressions of phospho-ERK1/2 in cells exposed to different bile acid for 3 min (P <0.05) (Figure 7). No expression of PPARγ in normal human esophageal epithelial cells, either exposed to acid/bile acid or not, was observed.

DISCUSSION

Increased cell proliferation has been shown in reflux esophagitis and BE[6]. Episodic exposure to acid has been found to increase *in vitro* cell proliferation in organ culture of BE[7] and in Barrett's-associated adenocarcinoma cell line (SEG-1)[8]. Moreover, it was reported the exposure to GCDC for 20 min also increased cell proliferation in SEG-1 cell line[9]. These results suggest that the refluxates probably result in the development of esophageal inflammation, intestinal metaplasia and malignant

Table 1 Effect of acid and bile acid on cell cycle

	3 min		6 min		12 min	
	S (%)	A (%)	S (%)	A (%)	S (%)	A (%)
pH 4.0	32.94±1.87[a]	0.89±0.08	24.12±2.08[a]	2.89±0.11[a]	21.77±1.53[a]	3.50±0.16[a]
pH 5.0	35.51±1.93[a]	0.73±0.05	26.68±1.91[a]	1.70±0.09[a]	25.53±1.72[a]	1.98±0.09[a]
pH 6.0	33.28±2.05[a]	0.85±0.07	27.83±1.06[a]	1.56±0.15[a]	25.49±1.95[a]	1.63±0.10[a]
GC	33.12±1.51[a]	0.92±0.05	35.03±2.06[a]	0.82±0.10	36.83±1.95[a]	0.71±0.09
TC	35.44±2.11[a]	0.76±0.09	37.05±2.14[a]	0.90±0.10	36.96±2.04[a]	0.80±0.11
CA	34.89±2.56[a]	0.88±0.09	34.59±1.97[a]	0.73±0.07	37.04±1.81[a]	0.93±0.08
DCA	33.37±1.64[a]	0.81±0.07	34.21±1.98[a]	0.85±0.16	36.57±1.82[a]	0.79±0.14
Control	29.61±1.82	0.64±0.10	30.56±1.12	0.71±0.09	32.58±1.89	0.58±0.13

S: S phase; A: Apoptosis; [a]$P < 0.05$.

progression by inducing abnormal proliferation. Since there have been some problems unsettled in the primary culture of human normal esophageal epithelial cells, the previous studies were mainly limited to the cells in pathologic status. Based on the newly-established primary culture technique of human normal esophageal cells[5], we observed, probably for the first time, the effects of acid and bile acid on cell proliferation in human normal esophageal cells.

We found an acid-pulse (3 min) enhanced proliferation in normal esophageal epithelial cells, whereas continuous acid exposure blocked cell proliferation. A brief acid exposure (3 min) increased proliferation ratios at 24 h, yet longer exposure (>6 min) led to decreased cell number, which was more significant at pH<5.0. This was further confirmed by the results of cell cycle determination, a brief exposure (3 min) to acid produced an increase of cells in S phase, while longer exposure (6, 12 min) decreased the cell proportion in S phase accompanied by an increased apoptosis percentage.

The above results show that the effects of acid on esophageal cells are time-dependent, the brief exposure has pro-proliferation effect and longer exposure can induce apoptosis. Previous reports have showed the effect of acid on apoptosis, exposure to pH 3.5 for 2 d led to a decreased cell number in mouse esophageal keratinocytes[10], and normal human esophageal epithelial cells could not survive in media below pH 5.5 for 12 h[11]. Esophageal acid reflux is judged clinically by pH < 4. Since cells are cultured *in vitro* with a neutral pH of 7.2-7.4, their tolerance to acid is limited; this is mainly due to the defect of tissue organization, blood transportation and mucosal barrier *in vivo*.

To identify whether bile acid has the similar pro-proliferation effect, we exposed the normal esophageal cells to 6 conjugated bile acids and 2 unconjugated bile acids of 250 μmol/L for 3-60 min. The concentration choice was based on median bile acid concentration found in esophageal aspiration in patients with BE[12]. We found a brief bile acid exposure (3-12 min) also increased proliferation ratios at 24 h and the cell proportion in S phase, while with prolongation of exposure time to the acid and bile acids, especially DCA, the cell number decreased, suggesting that DCA is more toxic than other bile acids. Furthermore, we found that toxic effect of acid was higher compared to bile acid with the same exposure time.

In most cases, duodenogastroesophageal reflux is the mixed reflux of acid and bile. The toxic effects of bile acids are known to vary with pH. Unconjugated bile acids (pKa>4) and glycine conjugates (pKa>6) are soluble in nonionic pattern when pH > 4, whereas taurine conjugates are freely soluble even at pH 2[12]. Bile acids in nonionic pattern are easy to enter cells because of their affinity to lipid. Esophageal perfusion studies in animal models showed that unconjugated bile acids caused mucosal damage selectively in alkaline solutions, whereas taurine conjugates were toxic in acidic conditions[13]. We investigated the influence of pH on the effect of bile acid in cells, and found that compared with bile acid exposure group, the mixed exposure group had a decreased proliferation ratio, which dropped to the lowest at pH 4, yet for GC the lowest proliferation ratio was seen at pH 6.5. So, we could conclude that acid aggravates the toxic effect of bile acid, which may be due to the influence of pH on the ionization degree of taurine conjugates and glycine conjugates. In addition, it may be also related to the synthetic toxic effects of acid and bile acid, since we found that with the same exposure time (3 min), acid or bile acid exposure enhanced cell proliferation, but the mixed exposure decreased the proliferation.

Since acid and bile reflux in esophagus have been clinically shown to be intermittent and repeated, the abnormal proliferation induced by acid and bile acid may play an important role in the development of reflux-related diseases. Though there have been various points of view about the damage mechanisms of refluxates, their pro-proliferative mechanism has been seldom reported. So, we investigated the relation between ERK signaling pathway and the proliferation effects of acid and bile acid. ERK is an important signal transduction factor, unreactive in cytoplasm in normal condition, can be activated by physicochemical stimulus to enter nucleus and is implicated in cell physiological and pathological processes, such as growth, division, proliferation, apoptosis and malignant progression[14]. We found the proliferation induced by acid and bile acid in normal esophageal epithelial cells was related to the up-regulation of ERK expression, which suggest that acid and bile acid may result in esophageal inflammation, intestinal metaplasia and malignant progression by inducing ERK-mediated proliferation.

Recently some reports have suggested the implication

of PPARγ in control of tumor cell growth and its possible role in the downstream of ERK signaling pathway[3,4]. PPARγ is over-expressed in many malignant tissues, but its expression in esophagus remains controversial. Some authors reported PPARγ mRNA level was lower in esophageal squamous cell carcinoma tissues than that of normal esophageal mucosa[3]. Others found PPARγ mRNA was expressed in biopsies of both normal esophageal mucosa and BE, but expression of this protein was detected only in BE[15]. In our study, no expression of this protein was observed either in normal esophageal epithelial cells or the cells exposed to acid or bile acid.

In conclusion, we have shown, probably for the first time, that a brief exposure to acid or bile acid induces *in vitro* proliferation in normal human esophageal epithelial cells, accompanied with the up-regulation of ERK, a potential molecular pathway whereby duodenogastroesophageal reflux may contribute to the development of reflux esophagitis, BE and esophageal adenocarcinoma. Moreover, acid aggravates the toxic effect of bile acid, which supports the view that the mixed reflux facilitates damage to the esophageal mucosa.

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RAPID COMMUNICATION

Antisense angiopoietin-1 inhibits tumorigenesis and angiogenesis of gastric cancer

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Abstract

AIM: To investigate the effect of angiopoietin-1 (Ang-1) on biological behaviors *in vitro* and tumorigenesis and angiogenesis *in vitro* of human gastric cancer cells.

METHODS: Human full-length Ang-1 gene was cloned from human placental tissues by RT-PCR method. Recombinant human Ang-1 antisense eukaryotic expression vector was constructed by directional cloning, and transfected by lipofectin method into human gastric cancer line SGC7901 with high Ang-1 expression level. Inhibition efficiency was confirmed by semi- quantitive PCR and Western blot method. Cell growth curve and cell cycle were observed with MTT assays and flow cytometry, respectively. Nude mice tumorigenicity test was employed to compare *in vitro* tumorigenesis of cells with Ang-1 suppression. Microvessel density (MVD) of implanted tumor tissues was analyzed by immunohistochemistry for factor VIII staining.

RESULTS: Full-length Ang-1 gene was successfully cloned and stable transfectants were established, namely 7Ang1- for antisense, and 7901P for empty vector transfected. 7Ang1- cells showed down-regulated Ang-1 expression, while its *in vitro* proliferation and cell cycle distribution were not significantly changed. In contrast, xenograft of 7Ang1- cells in nude mice had lower volume and weight than those of 7901P after 30 days' implantation ($P < 0.01$, 293.00 ± 95.54 mg *vs*. 624.00 ± 77.78 mg) accompanied with less vessel formation with MVD 6.00±1.73 compared to 7901P group 8.44±1.33 ($P < 0.01$).

CONCLUSION: Ang-1 may play an important role in tumorigenesis and angiogenesis of gastric cancer, and targeting its expression may be beneficial for the therapy of gastric cancer.

INTRODUCTION

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), two main members of angiopoietins family, are involved in both physiological and pathological angiogenesis processes. Ang-1 has been identified as a major activator of the tyrosine kinase receptor Tie2, leading to receptor autophosphorylation on binding. Ang-1 also stimulates endothelial cell migration *in vitro*[1,2]. Ang-2 is the naturally occurring antagonist to Ang-1 and inhibits Ang-1 mediated Tie2 phosphorylation; this effect leads to vessel destabilization, a necessary step in the initiation of angiogenesis[3,4].

Ang-1 and Ang-2 have been reported to be involved in several kinds of cancers, while their roles in gastric cancer progression and angiogenesis are still not fully understood. There have been several reports declaimed the effect of Ang-1 on tumorigenesis of human astrocytomas[5,6], breast cancer[7] and colorectal adenocarcinoma[8]. Previous report has identified that Ang-2 plays an important role in gastric cancer angiogenesis and progression[9,10], while little is known about the roles of Ang-1 in gastric cancer.

In the present study, we aimed to investigate the effect of Ang-1 on the biological behaviors and tumorigenesis and angiogenesis of human gastric cancer cells by modifying Ang-1 expression in SGC7901 gastric cancer cells by *in vivo* and *in vitro* examinations. Our results revealed that inhibition of Ang-1 expression would retard gastric cancer angiogenesis and progression.

MATERIALS AND METHODS

Tissue specimens and cell lines

Fresh placental tissue was obtained from the Department of Gynaecology and Obstetrics, Xijing Hospital, Xi'

an, China, with informed consent from the patients. Human gastric cancer cell line SGC7901 with high Ang-1 expression was preserved in our institute and cultured in RPMI1640 supplemented with 100 mL/L bovine serum.

Ang-1 antisense eukaryotic expression vector was conducted by RT-PCR method and directional cloning. Total RNA of fresh human placental tissue was extracted with Trizol (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. About 1 μg of total RNA was used for first strand cDNA synthesis according to the manufacturer's instructions. The full-length human Ang-1 cDNA was cloned using primer pairs: 5'-gaggggggaaagagtcaaacaaac-3' and 5'-cttgaccgtgaatctggagcc-3'. PCR parameters were 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and 72 ℃ for 2 min for 30 cycles, and the product was verified by 8 g/L gel electrophoresis. Sequence of PCR product was verified by the ABI PRISM 377 DNA Sequencer (Sangon, Shanghai, China). After PCR, the 1.9-kb fragment was cloned into the pMD18-T vector (Takara, Dalian, China) followed by *Hind*Ⅲ and *BamH* Ⅰ digestion and ligated into pcDNA3.1-V_5-His C expression vector (Invitrogen, Carlsbad, CA) named as pcDNA3.1-Ang1-. The sequence was confirmed by sequencing analysis.

Cell transfection

SGC7901 cells were plated and grown to 80% confluency without antibiotics. Stable transfections of pcDNA3.1-Ang1- and empty vector, pcDNA3.1-V_5-His C, were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. Transfected cells were selected with 300 mg/L G418 after 48 h. Clones were picked and expanded for an additional 2 months. Efficiency of Ang-1 suppression was confirmed by RT-PCR and Western blot methods.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was employed to compare the gene expression in different transfectants. The number of optimal replications was determined based on the linear correlations between cycle numbers and PCR products. Gene expression was presented by the relative yield of the PCR product from target sequences to that from GAPDH gene as control. PCR primers and reaction parameters were chosen according to the literature [11] and listed as follows: Ang-1, 5'-ACTGTGCAGATGTATATCAAG C-3' and 5'-GTGGAATCTGTCATACTGTGA A-3'; GAPDH, 5' -TGGGTGTGAACCATGAGAAGTA-3' and 5'-CGCTGTTGAAGTCAG AGGAGA-3'. The PCR parameters were 94℃ for 1 min, annealing at 60℃ for 1 min, and 72℃ for 1 min for 32 cycles for Ang-1 and 94℃ for 30 s, annealing at 52℃ for 30 s, and 72℃ for 45 s for 32 cycles for GAPDH.

Western blot analysis

Equal amounts of the extracted protein of cells were separated by 100 g/L SDS–PAGE; The protein bands were electro-transferred to nitrocellulose membrane. Expression of Ang-1 was analyzed using primary antibody (kindly provided by Micheal Hanner from Renegeron Pharmaceuticals, USA), followed by corresponding second antibody (Zhongshan, Beijing, China), then visualized by enhanced chemiluminescence (ECL, Amersham–Pharmacia Biotech, Beijing, China). Relative protein levels were calculated to β-actin as standard.

MTT assay

Exponentially growing cells were harvested and suspended in 96-well flat-bottomed plates (200 μL /well) (Costar). After incubation for 1, 3, 5 and 7 d, respectively, 20 μL of MTT solution (5 g/L) (Sigma, St louis, MO) was added to each well and further incubated for 4 h. One hundred and fifty μL of dimethylsulfoxide was added before absorbance at 490 nm was measured with a microplate reader BP800 (BIOHIT).

Cell cycle analysis

To evaluate whether the inhibition of Ang-1 would exert any effect on the cell cycle distribution of SGC7901 cells, we performed cytofluorimetric analysis. Briefly, cells were fixed with 70% ice-cold ethanol and stored at 4°C overnight, washed with PBS, and stained with propidium iodide (PI) (50 mg/L) for 30 min. Cell cycle histograms were generated after analysis of PI-stained cells by fluorescence activated cell sorting (FACS) with a Becton-Dickinson FACScan.

In vivo tumorigenicity

To investigate whether or not Ang-1 suppression would alter the tumorigenicity of SGC7901 cells, nude mice bred in our animal facilities were used with body weight range from 18 to 22 kilograms. Four $\times 10^6$ cells were injected subcutaneously into the right flank of nude mice. The experiment was performed on five mice in each group. The mean tumor volume was weekly measured and calculated according to the formula: $a \times (b)^2 \times 0.5$ (a = largest diameter, b = perpendicular diameter). All mice were sacrificed after 30 d and the subcutaneous tumor graft were surgically excised from the mice, fixed with formalin and paraffin-embedded 4-μm sections were prepared for immunohistochemical analysis.

Immunohistochemistry

Factor VIII staining was used to identify the microvessels in the tumor tissues by immunohistochemical method as usual. Briefly, after paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol, sections were incubated in 3 mL/L H_2O_2 to block endogeneous peroxidase activity. Each slide was incubated with normal goat serum for 20 min at room temperature, then rabbit anti-factor VIII related antigen antibody (Dako, MedBio Ltd, New Zealand) diluted at 1∶100 was applied on sections and incubated overnight at 4℃. After incubation with biotinylated goat anti-rabbit IgG (dilution 1:200) for 30 min at 37℃, each slide was rinsed in phosphate-buffered saline and was incubated in the avidin-biotin peroxidase complex for 30 min at 37℃. The peroxidase was visualized with 3-3'-diamino- benzidinetetrahydro chloride (DAB) solution and then counterstained with hematoxylin.

MVD evaluation

MVD was assessed according to the international

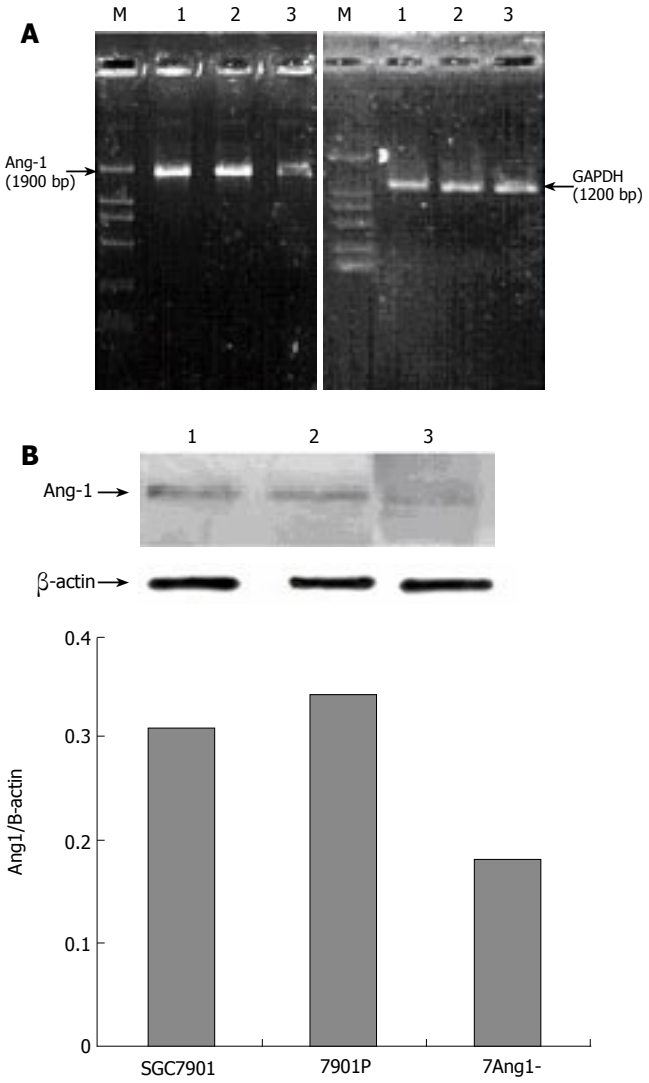

Figure 1 Verification of suppressive efficiency in parental and transfected cells. **A**: semi-quantitive RT-PCR. **B**: western blot. M: DL2000 marker; 1: SGC7901; 2: 7901P; 3: 7Ang1-.

consensus report[12]. Immuno stained slides were scanned at ×100 magnification to identify the areas with the highest number of vessels (so called "hot spot"). Counts were performed on five fields in the hot spot by two independent pathologists at ×200 magnification and the mean was taken and analyzed.

Statistical analysis

Statistical analysis was performed using SPSS software (version 10.0, SPSS Inc, Chicago). The ANOVA test was used to compare the differences between groups for MVD, tumor weight and volume. Differences were considered statistically significant at $P<0.05$.

RESULTS

Construction of Ang-1 antisense eukaryotic expression vector

Full length Ang-1 gene was amplified from placental tissues and cloned into pMD 18-T vector, then cut by by *Hind*Ⅲ and *BamH* Ⅰ digestion which would ensure inverse directional ligation into pcDNA3.1-V5-His C expression vector. The combined antisense expression vector named as pcDNA3.1-Ang1- was verified as expected by sequencing.

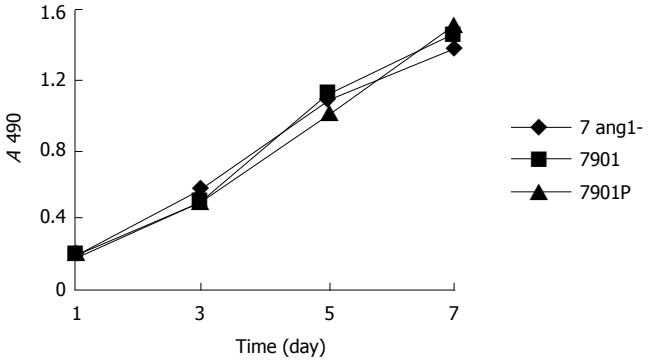

Figure 2 Proliferation of SGC7901-derived cells.

Transfectants of SGC7901-derived cells

Stable transfectants of 7901-derived cells were obtained by Lipofectamine 2000 transfection, named 7Ang1- for pcDNA3.1-Ang1- and 7901P for empty vector-transfected cells. We could clearly find that moderate targeted-inhibition effect was detected in 7Ang1- cells at both mRNA (Figure 1A) and protein (Figure 1B) levels, in contrast, control experiment with the empty vector alone showed a negligible effect on Ang-1 expression (Figure 1).

Proliferation and cell cycle of SGC7901-derived cells

To determine if interference of Ang-1 expression has an effect on the proliferation and cell cycle distribution of SGC7901 cells, MTT assay and FACS method were utilized, respectively. According to Figure 2, the proliferation of 7Ang1- cells was not significantly changed compared to that of 7901P cells. In addition, cell cycle distribution of Ang1- and 7901P were 71.9% and 59.2% for G_1 phase, 15.3% and 12.5% for G_2 phase, 25.5% and 15.6% for S phase, 0.328 and 0.281 for the proliferation index, respectively. Taken together, it suggests that interfering Ang-1 has no direct effect on *in vitro* proliferation and cell cycle of gastric cancer cells.

In vivo tumorigenicity of SGC7901-derived cells

Xenograft model was employed to compare the tumorigenicity of SGC7901 cells before and after Ang-1 inhibition. Subcutaneous tumor node of different groups became palpable almost simultaneously after 7d transplantation. Tumor from 7Ang1- cells appeared to grow slower than those from 7901P cells after 16 d (Figure 3). Finally, tumor grafts were collected and weighed after 30 d, and tumor tissues derived from 7Ang1- cells showed significantly decreased weight compared to those from 7901P cells with mean tumor graft weight (mg) being 293.0±95.5 for 7Ang1- and 624.0±77.8 for 7901P cells ($P<0.01$).

To further elucidate whether reduced angiogenesis account for the suppressed *in vivo* growth of 7Ang1- cells, MVD was assessed by immunohistochemistry. As shown in Figure 4, microvessels could easily be observed by factor

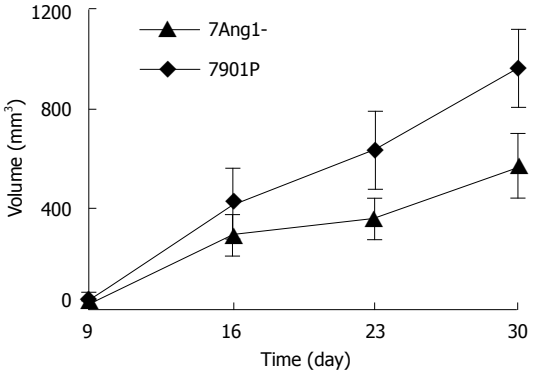

Figure 3 *In vivo* growth of tumor xenograft.

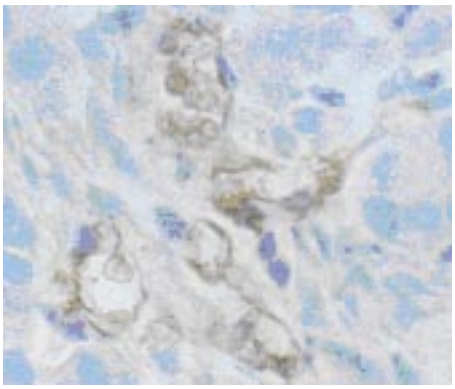

Figure 4 Factor VIII positive tumor microvessels (SABC x 200).

VIII staining. Statistics analysis showed a significantly less MVD was present in 7Ang1- group 6.0±1.7 compared to 8.4±1.3 in 7901P group ($P<0.05$). This result indicated that suppressed *in vivo* tumorigenicity of 7Ang1- cells might be mediated through reduced angiogenesis.

DISCUSSION

Gastric cancer remains a common malignancy in many countries of the world, especially in Asia, and is still among the most important causes of cancer-related death worldwide[13]. Its conventional treatment includes surgery, radiation and chemotherapy. Nowadays, increasing evidence has shown that angiogenesis is essential for the growth of solid tumor and tumor angiogenesis research has become one of the most active fields for anticancer therapies.

Several studies suggested that VEGF receptor pathway and Tie2 pathway are independent and essential mediators of angiogenesis and both play important roles in the tumor angiogenesis[14,15]. Tie2 is a novel endothelial cell-specific molecule involved in both physiological and pathological processes. Tie2 is required for normal vascular development perhaps via the regulation of vascular remodeling and endothelial cell interactions with supporting pericytes and smooth muscle cells[3]. Previous report found that inhibition of Tie2 using a kinase-deficient Tie2 construct or an adenoviral vector delivering a recombinant single-chain antibody fragment into body would inhibit the growth of human tumor xenografts[16,17].

Four ligands for the Tie2 receptor have been identified so far, named Ang-1 to -4. Among them, Ang-1 and -2 were mainly associated with tumor angiogenesis[18]. The findings from functional study of Ang-2 in several types of tumor showed that Ang-2 could stimulate tumor angiogenesis, thus promoting tumor progress[9,19,20], while those from Ang-1 showed great heterogeneity. For example, Zadeh *et al*[6] found that Ang-1 increased the vascular growth of both subcutaneous and intracranial xenografts of astrocytoma; also, Ang-1 promoted tumor angiogenesis and tumor vessel plasticity of human cervical cancer in mice[21]. In contrast, others have reported that Ang-1 could inhibit angiogenesis and growth of hepatic and colon cancer[22] and suppress breast cancer xenograft angiogenesis by blocking tumor neovasculature[7]. Taken together, it suggests that the role of Ang-1 in tumor progression may be context-dependent.

Our previous study found that Ang-1 was highly expressed in SGC7901 cells[23], while its expression in 7Ang1- cells was effectively reduced using antisense method in the present study. Further study revealed that the biological behaviors including proliferation and cell cycle of 7Ang1- cells were not significantly changed, so it seemed that interfering Ang-1 could not alter *in vitro* growth of SGC7901 cells directly; this is similar to other studies[9] in which growth of the gastric cancer cells was not affected by transfection with Ang-2.

Tumorigenicity assay showed that tumor xenograft of 7Ang1- group grew slower as their volume and weight were smaller than those from control group. MVD assessment showed significantly decreased angiogenesis in tumor from 7Ang1- group, indicating inadequate blood supply might account for suppressed *in vivo* growth. To our best knowledge, this is the first direct evidence for the role of Ang-1 in gastric cancer angiogenesis. However, in initial stage of tumor progression, two groups did not show significant difference, and until 16 d after transplantation the suppressive effect appeared dramatic when tumor grew larger. This result is consistent with our previous study in which suppressing the expression of VEGF in SGC7901 cells did not exert any significant effect on its proliferation but restricted its *in vivo* growth in nude mice[24]. Collectively, it infers that suppressing either VEGF or Ang-1 cannot prohibit the onset of tumor, but may slow *in vivo* growth of tumor cells by inhibiting angiogenesis. It was reported that Ang-2 was implicated in angiogenesis and progression of gastric carcinoma via induction of proteases such as MMP-1 and -9[9]. Combining result from our present and previous studies that significant expression of Ang-1 and Ang-2 was found in human gastric cancer tissues and cell lines[23], it infers that both Ang-1 and -2 participate in the angiogenesis and progression of gastric cancer. Previous studies indicated that Ang-1 induced the secretion of MMP-2 and small amounts of proMMP-3 and proMMP-9 in endothelial cells[25], whether or not this effect is involved in the mechanism of Ang-1 contribution to gastric cancer and progression is still unclear, however it still could be concluded from our present study that stimulating angiogenesis by Ang-1, in part at least, could promote tumor progress.

In summary, Ang-1 may contribute to the progression of gastric cancer and inhibiting its expression in gastric

cancer cells could suppress *in vivo* tumorigenicicy. It could also be a useful therapeutic target to prevent gastric cancer or inhibit its malignant progression. Tumor angiogenesis is a multi-factor associated process, and recent study showed that simultaneous phenotypic knockout of VEGF-R2 and Tie2 with an intradiabody could effectively reduce tumor growth and angiogenesis *in vivo*[26]. It suggests that combination of targeting main angiogenesis modulators including VEGF, Ang-1 and Ang-2 and their corresponding receptors can be beneficial for tumor therapeutics.

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RAPID COMMUNICATION

Increase of $CD4^+CD25^+$ T cells in $Smad3^{-/-}$ mice

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Abstract

AIM: To investigate the changes of lymphocyte subpopulations, especially $CD4^+CD25^+$ T regulatory cells in $Smad3^{-/-}$ mice.

METHODS: Hematological changes and changes of lymphocyte subpopulations were detected in $Smad3^{-/-}$ mice using cell counter and flow cytometry, respectively, and compared to their littermate controls.

RESULTS: The numbers of neutrophils and lymphocytes in peripheral blood were significantly increased in $Smad3^{-/-}$ mice compared to littermate controls. $CD19^+$ expressing cells in blood and spleen, and $CD8^+$ T cells in thymus were all markedly decreased in $Smad3^{-/-}$ mice. More important, $Smad3^{-/-}$ mice had an increased population of $CD4^+CD25^+$ T cells in peripheral lymphoid tissues, including thymus, spleen, and lymph nodes.

CONCLUSION: These observations suggest that the changes of lymphocyte subpopulations might play a role in susceptibility to inflammation of $Smad3^{-/-}$ mice.

Key words: $CD4^+CD25^+$ T cells; Lymphocyte subpopulation; SMAD3; TGF-β signaling

Wang ZB, Cui YF, Liu YQ, Jin W, Xu H, Jiang ZJ, Lu YX, Zhang Y, Liu XL, Dong B. Increase of $CD4^+CD25^+$ T cells in $Smad3^{-/-}$ Mice. *World J Gastroenterol* 2006; 12(15):2455-2458

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INTRODUCTION

TGF-β plays an important role in maintaining immune homeostasis. It signals through a set of transmembrane receptor serine/threonine kinases unique to the large superfamily of TGF-β related proteins [1]. As a downstream cytoplasm signaling element of TGF-β receptors, Smad3 mediates a positive signal pathway from the receptor serine/threonine kinases to the nuclei [2]. Previous reports revealed Smad3 plays an important role in mediating TGF-β signal in T lymphocytes and neutrophils, and demonstrated that Smad3 deficiency results in immune dysregulation and susceptibility to opportunistic infection [3].

The immune system discriminates between self and non-self, establishing and maintaining unresponsiveness to self. There is clear evidence that clonal deletion of self-reactive T and B cells is a major mechanism of self-tolerance [4]. However, the fact that potentially hazardous self-reactive lymphocytes are present in the periphery of normal adult individuals [5] reveals that the mechanisms that can prevent pathological autoimmunity exist. In recent years, a burst of papers are focused on a population of $CD4^+$ T cells that constitutively express the IL-2Rα (CD25) T cells and reveal them as key "actors" to self-tolerance[6,7]. A direct experiment to assess the regulatory role of $CD4^+CD25^+$ T cells in self-tolerance reported that the adoptive transfer of $CD4^+CD25^+$-depleted T cells could induce several organ-specific autoimmune diseases in immunodeficient animals [8]. $CD4^+CD25^+$ T cells also regulate antibody responses against self- and non-self-antigens by direct inhibitory effects on B cells or via inhibition of Th cell differentiation [9, 10]. In addition to self-tolerance and autoimmunity, there is evidence that $CD4^+CD25^+$ T cells are actively engaged in negative control of a broad spectrum of immune responses induced by microbial infection [11-13]. They can also mediate transplantation tolerance [14] and maternal tolerance to the foetus [15].

Although great progress in $CD4^+CD25^+$ T cells study has been made in recent years, many issues remain to be solved. For example, the involvement of TGF-β in $CD4^+CD25^+$ T cell immunoregulatory function is still controversial [16-19]. In the present study, we examined the changes of lymphocyte subpopulations in peripheral lymphoid tissues of $Smad3^{-/-}$ mice as well as their controls. Our results showed that $Smad3^{-/-}$ mice were associated with an increased population of $CD4^+CD25^+$ T cells, suggesting that $CD4^+CD25^+$ T cells might play a role in susceptibility to inflammation of $Smad3^{-/-}$ mice.

MATERIALS AND METHODS

Mice

$Smad3^{-/-}$ mice were generated by targeted gene disruption

in murine embryonic stem cells by homologous recombination [3]. Both Smad3$^{-/-}$ mice and their littermate controls (wild-type, Smad3$^{+/+}$) were provided by Xiao Yang (Institute of Biotechnology, Beijing, China). The mice used in these experiments were 6-8 wk of age.

Antibodies and reagents

PE-anti-CD4, FITC-anti-CD8, FITC-anti-CD3, and PE-anti-CD19 were purchased from Southern Biotechnology Associates (Birmingham, USA). FITC-anti-CD25 was purchased from Biolegend (San Diego, CA).

Analysis of leukocytes in peripheral blood

Before mice were sacrificed, approximately 20 μL blood samples were collected through tail vein, diluted, and then analyzed on Sysmex F-820 semi-automatic analyzer (Japan).

Flow cytometry of lymphocytes

Peripheral blood, thymus, spleen and lymph nodes were harvested from mice. Single-cell suspensions were subjected to hypotonic lysis of red blood cells (Becton Dickinson), washed in phosphate-buffered saline, stained with fluorescein-conjugated antibodies according to standard protocols, and then analyzed on an FACScan (Beckman Dickinson). For isolation of peripheral blood mononuclear cells (PBMC), 2 mL of heparinised peripheral blood diluted 1:1 with PBS was layered onto an equal volume of Ficoll-Hypaque density gradient solution and centrifuged at 300 r/min at room temperature. The mononuclear cells were collected, washed twice with PBS.

Statistical analysis

Difference was defined as being statistically significant when P <0.05 was obtained using Student's t test.

RESULTS

Increased numbers of neutrophils and lymphocytes in Smad3$^{-/-}$ mice

We first compared the total numbers of white blood cells and differential distributions of leukocytes in peripheral blood samples from Smad3$^{-/-}$ mice and littermate control mice. A marked increase in absolute white blood cell counts was observed in Smad3$^{-/-}$ mice (P < 0.01). Accordingly, the numbers of neutrophils and lymphocytes were also elevated in Smad3$^{-/-}$ mice compared to their controls (Figure 1). These results are consistent with a previous report that Smad3$^{-/-}$ mice exhibited invasive mucosal infection involving multiple immune organs [3].

Changes of lymphocyte subpopulations

Susceptibility of Smad3$^{-/-}$ mice to infection and tissue inflammation [3] made us wonder whether quantitative changes of lymphocytes were present in these mice. The results showed that numbers of CD19^{+}-expressing cells (most B cells) in the peripheral blood and spleen were significanty decreased in Smad3$^{-/-}$ mice compared to their controls (Figures 2A and 2C). In addition, the number of CD8^{+} T cells was also reduced in thymus in Smad3$^{-/-}$ mice (Figure 2B). Analysis of lymph nodes did not reveal any significant difference between the mutant mice and littermate controls (Figure 2D).

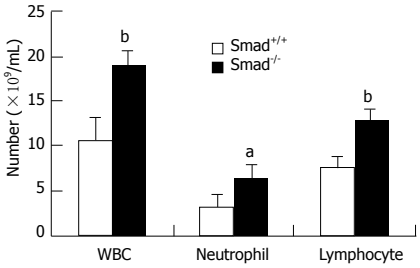

Figure 1 Total numbers and differential distributions of blood leukocytes in Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. Peripheral blood samples were collected from tail veins of mice, and then analyzed on Sysmex F-820. Shown here are the means and standard deviations of total numbers and distributions of blood leukocytes from 4 wild-type and 4 mutant mice (^{a}P < 0.05, ^{b}P < 0.01).

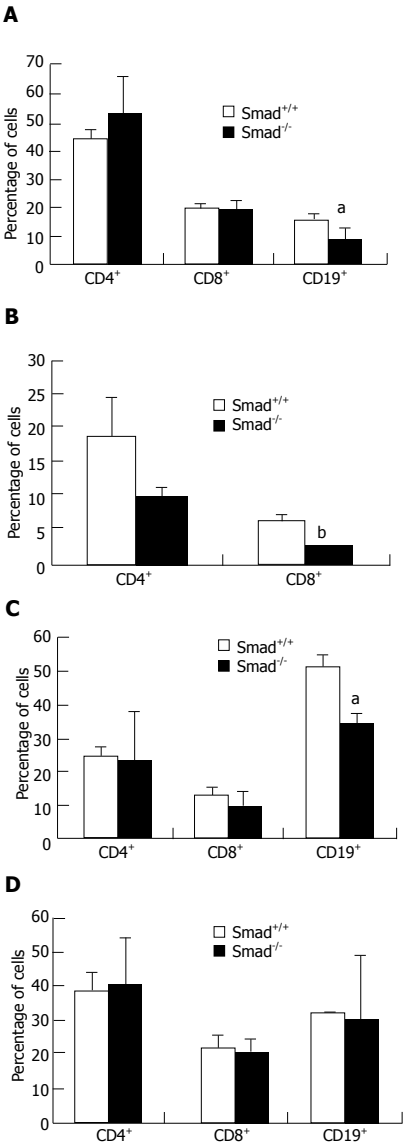

Figure 2 Percentage of lymphocyte subpopulations in peripheral blood (A), thymus (B), spleen (C) and lymph nodes (D) of wild-type and mutant mice. The cells were stained with PE-anti-CD4 and FITC-anti-CD8, or with FITC-anti-CD3 and PE-anti-CD19, and then subjected to cytometric analyses. Shown here are the means and standard deviations of percentage of lymphocyte subpopulations from 4 wild-type and 4 mutant mice (^{a}P < 0.05, ^{b}P < 0.01).

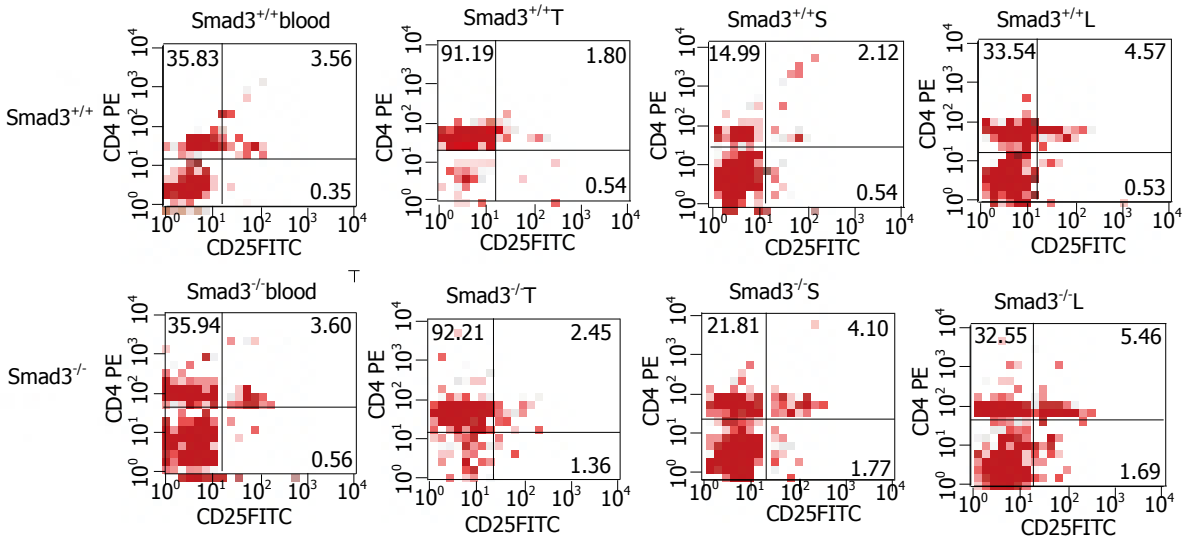

Figure 3 Percentage of CD4$^+$CD25$^+$ T cells in peripheral lymphoid tissues of Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. PBMC (blood) or single-cell suspensions from thymus (T), spleen (S), and lymph nodes (L) were prepared according to "MATERIALS AND METHODS", co-labeled with PE-anti-CD4 and FITC-anti-CD25, and then analyzed by FACScan.

Increased CD4$^+$CD25$^+$ T cells in Smad3$^{-/-}$ mice

CD4$^+$CD25$^+$ T cells play an important role in maintaining the equilibrium between immunity and tolerance[20,21]. Many papers have reported that this population of cells is able to suppress proliferation and effector function of CD4$^+$ and CD8$^+$ T cells [22-24]. To explore whether the decreased lymphocytes in Smad3$^{-/-}$ mice were related to the CD4$^+$CD25$^+$ T cells, we examined this population of cells in peripheral lymphoid tissues of Smad3$^{-/-}$ mice and littermate controls. Smad3$^{-/-}$ mice exhibited a greater percentage of CD4$^+$CD25$^+$ T cells in thymus, spleen and lymph nodes, compared to controls. In peripheral blood, however, no difference was observed between mutant mice and wild type in regarding to CD4$^+$CD25$^+$ T cell proportion (Figure 3).

DISCUSSION

TGF-β is an essential endogenous regulator of T-cell function [25]. It has been recently reported that TGF-β$^{-/-}$ mice have normal numbers of CD4$^+$CD25$^+$ T cells after birth, indicating that CD4$^+$CD25$^+$ T cells are able to develop in complete absence of endogenous TGF-β expression [16, 17]. This made us think whether quantitative or functional changes of CD4$^+$CD25$^+$ T cells occurred in Smad3$^{-/-}$ mice. Our main finding in this study is that Smad3$^{-/-}$ mice had increased CD4$^+$CD25$^+$ T cells compared to their littermate controls (Figure 3). Our results showed that neutrophil and lymphocyte numbers increased (Figure 1) and that lymphocyte subpopulation decreased in the peripheral lymphoid tissues of Smad3$^{-/-}$ mice (Figure 2), which are consistent with the previous reports [3,26].

During infection, the balance between self-reactive effector T cells and regulatory T cells could determine the time of onset, the intensity and duration of autoimmune response [27]. Recent studies have focused on a population of CD4$^+$ T cells that constitutively express CD25. CD4$^+$CD25$^+$ T cells comprise 5%-10% of the peripheral CD4$^+$ T cell pool of normal mice and humans and exhibit immunosuppressive abilities both *in vitro* and *in vivo* [28, 29]. Studies of human diseases indicate that the functional CD4$^+$CD25$^+$ T cells are enriched in inflamed joints of patients with rheumatoid arthritis [30] or with the juvenile idiopathic arthritis [31]. In our study, we showed that an increased population of CD4$^+$CD25$^+$ T cells was present in Smad3$^{-/-}$ mice, which could partially account for the susceptibility to inflammation of these mutant mice. However, our results and those of a previous study [3] did not reveal any significant difference of CD4$^+$ T cells of spleen and lymph nodes between the asymptomatic mice and littermate controls. A possible explanation for the increase of CD4$^+$CD25$^+$ T cells is that they are derived from the CD4$^+$CD25$^-$ cells under the condition of Smad3 gene mutation. It is well accepted that CD4$^+$CD25$^+$ T cells can be generated by the activation of mature, peripheral CD4$^+$CD25$^-$ T cells under different stimulatory conditions [28]. Further studies should concentrate on defining the functional characteristics of the CD4$^+$CD25$^+$ T cells in Smad3$^{-/-}$ mice to gain a better insight into the mechanisms of susceptibility to inflammation.

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RAPID COMMUNICATION

Early removing gastrointestinal decompression and early oral feeding improve patients' rehabilitation after colorectostomy

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Abstract

AIM: To evaluate the feasibility, safety, and tolerance of early removing gastrointestinal decompression and early oral feeding in the patients undergoing surgery for colorectal carcinoma.

METHODS: Three hundred and sixteen patients submitted to operations associated with colorectostomy from January 2004 to September 2005 were randomized to two groups: In experimental group ($n = 161$), the nasogastric tube was removed after the operation from 12 to 24 h and was promised immediately oral feeding; In control group ($n = 155$), the nasogastric tube was maintained until the passage of flatus per rectum. Variables assessed included the time to first passage of flatus, the time to first passage of stool, the time elapsed postoperative stay, and postoperative complications such as anastomotic leakage, acute dilation of stomach, wound infection and dehiscense, fever, pulmonary infection and pharyngolaryngitis.

RESULTS: The median and average days to the first passage of flatus (3.0 ± 0.9 *vs* 3.6 ± 1.2, $P < 0.001$), the first passage of stool (4.1 ± 1.1 *vs* 4.8 ± 1.4 $P < 0.001$) and the length of postoperative stay (8.4 ± 3.4 *vs* 9.6 ± 5.0, $P < 0.05$) were shorter in the experimental group than in the control group. The postoperative complications such as anastomotic leakage (1.24% *vs* 2.58%), acute dilation of stomach (1.86% *vs* 0.06%) and wound complications (2.48% *vs* 1.94%) were similar in the groups, but fever (3.73% *vs* 9.68%, $P < 0.05$), pulmonary infection (0.62% *vs* 4.52%, $P < 0.05$) and pharyngolaryngitis (3.11% *vs* 23.23%, $P < 0.001$) were much more in the control group than in the experimental group.

CONCLUSION: The present study shows that application of gastrointestinal decompression after colorectostomy can not effectively reduce postoperative complications. On the contrary, it may increase the incidence rate of fever, pharyngolaryngitis and pulmonary infection. These strategies of early removing gastrointestinal decompression and early oral feeding in the patients undergoing colorectostomy are feasible and safe and associated with reduced postoperative discomfort and can accelerate the return of bowel function and improve rehabilitation.

Key words: Gastrointestinal decompression; Feeding; Colorectostomy

Zhou T, Wu XT, Zhou YJ, Huang X, Fan W, Li YC. Early removing gastrointestinal decompression and early oral feeding improve patients' rehabilitation after colorectostomy. *World J Gastroenterol* 2006; 12(15): 2459-2463

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INTRODUCTION

At present, gastrointestinal decompression after abdominal operations is still widely practiced in clinic. Routine use of nasogastric tubes after excision and anastomosis of digestive tract is aimed to hasten the return of bowel function, prevent pulmonary complications, diminish the risk of anastomotic leakage, and increase patients' comfort and shorten hospital stay. But nasal insertion of a gastric tube for prolonged gastrointestinal decompression causes extreme discomfort, and can be easily dislodged. Several complications of nasogastric intubation have been described, including sinusitis, injury to the vocal cords, and iatrogenic gastric perforation, nasal trauma, nasal hemorrhage, laryngeal injury, esophageal ulceration, gastroesophageal reflux, fluid and electrolyte imbalances, aspiration pneumonia, feeding dysfunction, and psychological problems[1-4]. Indeed, a unique randomized study by Hoffman *et al* reaffirms that patients find nasogastric tube more inconvenient and uncomfortable than gastrostomy tubes, even though gastrostomy tubes are left *in situ* for up to 4 wk[2]. Some researches have shown that the postoperative nasogastric tube could increase postoperative complications and furthermore, affect the

nutrient intake. Recent evidence seems to indicate that immediate postoperative feeding is feasible and safe after either laparoscopic surgery or laparotomy, including gastrointestinal tract surgery[5,6].

In the light of these evidence, we conducted a randomized study to evaluate the feasibility, and safety as well as the tolerance of early removing gastrointestinal decompression and early oral feeding in the patients undergoing colorectostomy for colorectal tumor in West China Hospital of Sichuan University from January 2004 to September 2005. It is hoped that this study will help to clarify strategies to accelerate postoperative recovery and to reduce patients' complications.

MATERIALS AND METHODS

Cases selection

Between January 2004 and September 2005, three hundred and sixteen consecutive patients receiving excision and anastomosis for colorectal tumor were entered the study. All patients were randomly chosen to undergo early and or late nasogastric tube removal. Nasogastric tubes were routinely placed following anesthetic induction. Patients were divided into two groups. In the experimental group, nasogastric tubes were removed within 12-24 h after the operation. The patients were provided immediately water and gradually to a liquid fiberless diet after one day, and a semi-liquid fiber diet after three days. In the control group, nasogastric tubes were removed upon report of passage of flatus by the patient, usually within three-five days after surgery.

Methods

A nasogastric tube was placed in all patients during operation. The tube was removed in control group with gastrointestinal decompression after passage of gas by intestines with continuous vacuum aspiration. The nasogastric tubes in the experimental group without gastrointestinal decompression were removed from 12 to 24 h after operation. Then, the patients were monitored prospectively, for time to first passage of flatus, time to first passage of stool, postoperative stay, and complications. Febrile morbidity was defined as two armpit temperatures greater than 38.5℃, taken at least 4 h apart, starting 24 h after operation. For the control group patients, we also recorded the time to removal of the nasogastric tube and the postoperative complications. Postoperative stay was calculated from the first postoperative day to the day of hospital discharge. Criteria for hospital discharge included: absence of nausea, vomiting, and abdominal distention; ability to tolerate oral feeding; spontaneous micturition; adequate healing, and absence of fever. Those suffering from anastomotic leaks were subjected to treatments such as anti-infective treatment, nutritional support or colostomy. Correspondingly, acute dilatation of stomach was subjected to placement of nasogastric tubes.

Statistical analysis

The χ^2 test was used for analysis of qualitative variables and Student's *t* test for continuous variables. Results of the two groups were compared using Student's *t* test. $P<0.05$ indicated significant difference. Analyses were performed using SPSS statistical software (SPSS for Windows Ver.11.5).

Table 1 Patient characteristics

General data	Experimental group (n = 161)	Control group (n = 155)
Gender(M/F)	92/69	83/72
Mean age (yr)	55.3±16.7	57.1±19.8
Range	21-78	24-85
Type of tumor		
Colon cancer	29	28
Rectal cancer	132	127
Type of operation		
Right hemicolectomy	17	15
Left hemicolectomy	12	13
Anterior rectal resection	132	127

RESULTS

General data

It was shown that there were no significant differences between two groups in terms of sex ($P>0.05$), and age ($P>0.05$) (Table 1). No significant difference was found between two groups in case distributions ($P=1.000$).

The time to first passage of flatus, stool and the length of postoperative stay and the incidence of complications after operation are shown in Table 2. The time to first passage of flatus was seen, on average, on postoperative day 3.0 in the early removing gastrointestinal decompression and early oral feeding group and on day 3.6 in the control group ($P<0.001$). The first defecation was 0.7 d sooner in the experimental group (postoperative day 4.1) than in the control group (4.8 d; $P<0.001$). The postoperative hospital stays for the experimental and control groups were 8.4±3.4 d and 9.6±5.0 d, respectively. The postoperative complications for the experimental group and control group were seen in 23 cases and 70 cases, respectively. Compared with the control group, the total incidence of complications in the experimental group was evidently higher ($P<0.001$). But the anastomotic leakage (1.24% *vs* 2.58%), acute dilation of stomach (1.86% *vs* 0.06%) and wound complications (2.48% *vs* 1.94%) were similar in the two groups. Symptoms as fever and leakage of intestinal contents were diagnosed as anastomotic leakage. Six cases suffered from the lesion in the two groups. All the leakages occurred during excision and anastomosis of lower or ultra-lower rectal tumor and healed after clinical therapy. Those who suffered from abdominal distension, emesis and succussion splash of stomach were diagnosed as acute dilatation of stomach and then subjected to gastrointestinal decompression. Two cases suffered from the wound infection and 2 cases suffered from the wound dehiscense in the experimental groups, and 3 cases suffered from wound infection but no wound dehiscense in the control group. The fever (3.73% *vs* 9.68%, $P<0.05$), pulmonary infection (0.62% *vs* 4.52%, $P<0.05$) and pharyngolaryngitis (3.11% *vs* 23.23%, $P<0.001$) were much more in the control group

Table 2 Clinical features and complications of experimental and control groups

Parameters	Experimental group	Control group (n = 161)	P value (n = 155)
Time to first passage of flatus (d)	3.0±0.9	3.6±1.2[b]	0.000
Time to first passage of stool (d)	4.1±1.1	4.8±1.4[b]	0.000
Postoperative stay (d)	8.4±3.4	9.6±5.0[a]	0.016
Total complication			
Anastomotic leakage (n%)	2(1.24)	4(2.58)	0.441
Acute dilation of stomach (n%)	3(1.86)	1(0.06)	0.623
Wound complication (n%)	4(2.48)	3(1.94)	1.000
Fever (n%)	6(3.73)	15(9.68)[a]	0.042
Pulmonary infection (n%)	1(0.62)	7(4.52)[a]	0.034
Pharyngolaryngitis (n%)	5(3.11)	36(23.2)[b]	0.000

[a]P < 0.05 *vs* experimental group; [b]P < 0.001 *vs* experimental group.

than in the experimental group. Eight cases of pulmonary infection were found in the two groups by chest X-ray and cured with anti-inflammatory therapy. Any symptom associated with throat discomfort or pain was diagnosed as pharyngolaryngitis; 23.23% of patients suffered from pharyngolaryngitis in the control group and only 3.11% in the experimental group.

DISCUSSION

There are several traditional dicta that are transmitted from generation to generation of surgeons but lack any clear scientific foundation. These include performing gastric decompression after abdominal surgery and delaying oral feeding until the resolution of ileus, as commonly observed in daily surgical practice. In China at present, 97.5% of surgeons routinely place and keep the nasogastric tube until the passage of gas through anus after excision and anastomosis of lower digestive tract, while 2.5% of surgeons discard gastrointestinal decompression 2-3 d after operation before the passage of gas through anus[7]. Indeed, the nasogastric tube can cause moderate to severe discomfort in 88%, severe discomfort in 70% of the patients and significantly delay the return of normal gastrointestinal function[8,9].

Recently, a meta-analysis shows that routine nasogastric decompression does not accomplish any of its intended goals. The analysis included 28 studies fulfilling the eligibility criteria and 4194 patients. It was found that those not having a nasogastric tube routinely inserted experienced an earlier return of bowel function, a marginal decrease in pulmonary complications, and a marginal increase in wound infection and ventral hernia. Anastomotic leakage was similar in the two groups. For this reason, the authors suggest that the routine nasogastric decompression should be abandoned in favor of selective use of the nasogastric tube[10].

We believe that the omission of routine postoperative gastrointestinal decompression may be an important first step in improving the rate of gastrointestinal recovery and shortening hospital stay. Anastomotic disruption after surgical intervention is an infrequent complication, and may lead to severe morbidity and mortality when it occurs. Of the various gastric procedures, the Roux-en-Y gastric bypass (RYGB) has one of the highest risks for anastomotic leakage. Consequently, a nasogastric tube is frequently placed when these operations are performed. But in Italy, a prospective multicenter randomized trial showed that routine placement of a nasogastric tube after Roux-en-Y oesophagojejunostomy is unnecessary in elective total gastrectomy for gastric cancer[11]. Huerta *et al* also suggest that routine placement of a nasogastric tube after RYGB is unnecessary; on the contrary, elimination of postoperative nasogastric decompression decreased postoperative fever and pulmonary problems, and improved patient comfort by decreasing sore throat and nausea[6,12]. In colorectal surgery, the average volume of gastric juice in the group with gastrointestinal decompression was 200 mL daily after operation. Because the total volume of digestive juice is 6000-10000 mL, the gastrointestinal decompression following excision and anastomosis of lower digestive tract can not reduce the pressure of gastrointestinal tract and has no obvious preventing effects on postoperative complications[7,13].

Compared with the control group, the removal of routine gastrointestinal decompression did not increase the postoperative probability of anastomotic leakage (P = 0.441), acute dilation of stomach (P = 0.623) and wound complication (P = 1.000). The data from the present study not only confirmed that placement of a nasogastric tube can be safely omitted in colorectostomy but also demonstrated that routine gastrointestinal decompression may increase the postoperative complications, such as the fever, pulmonary infection, pharyngolaryngitis.

Surgery is a traumatic procedure. The metabolic response of the body is a physiological mechanism that, according to the magnitude and duration of the event, can impact on the patient's morbidity and survival. Early enteral feeding can help to improve energy and protein intake, decrease the negative impact of the metabolic response to injury, stimulate motor, resorption, synthetic, and barrier functions of the small intestine, improve the return of gastrointestinal function and reduce the duration of postoperative ileus, and reduce the risk of serious complications[5,14,15]. Traditionally, tolerance of oral feeding is based on the passage of flatus. However, an in-depth review of the physiology of postoperative ileus suggested that such an approach is excessively conservative. It has been shown that paralysis of the small bowel is transient; the gastric paralysis lasts 24 h, and paralysis of the colon lasts 48–72 h[16]. The gastrointestinal tract motility of the patients undergoing major abdominal surgery is transiently impaired, leading to the so-called postoperative ileus (POI). It not only causes patient discomfort, but is also related to abdominal complications and worsening of the nutritional status, as well as increases length of hospital stay and costs[15].

POI is characterized by a transient impairment of bowel function and reduced motility sufficient to disrupt effective transit of intestinal contents[17-19]. It is a transient bowel dysmotility that occurs following abdominal surgery. Multiple factors are thought to contribute to the pathogenesis of POI, including physical manipulation

of the bowel, surgical stress and inflammatory mediators (including endogenous opioids), changes in electrolyte and fluid balance, neural reflex and inflammatory changes, pharmacologic agents such as inhalation anesthetics, and use of opioids for postoperative analgesia[20-22]. In animal experiments, it shows that the pathogenesis of postoperative gastric ileus induced by intestinal manipulation involves viscero-sympathetic pathways. Intestinal manipulation causes impaired gastric motility via inhibitory sympathetic efferent pathway. Feeding may improve the postoperative gastric motility[22]. Chan *et al*[23] suggest that metoclopramide not only prevents prolonged POI at an early postoperative stage, but also can be used as a safe prokinetic drug for post-operative intestinal dysmotility.

Oral intake after intestinal anastomoses has traditionally been prescribed only after the resolution of ileus. de Aguilar-Nascimento JE *et al*[24] suggest the return of oral feeding on the first postoperative day in patients submitted to intestinal anastomoses is safe, not associated with the occurrence of anastomotic dehiscence and moreover, related to a quicker resolution of ileus. A clinical trial showed that gastrografin not only facilitates early oral feeding but also reduces hospitalization after elective colorectal surgery. It can decrease bowel-wall edema and enhance bowel peristalsis[25]. Even, gum-chewing might be beneficial for short hospital stay because it can stimulate bowel motility and should be added as an adjunct treatment in postoperative care[26]. In our study, the time to first passage of flatus ($P < 0.001$), the time to first passage of stool ($P < 0.001$) and the time of postoperative stay ($P < 0.05$) were sooner in the experimental group than in the control group. Our study showed that early oral feeding can be administered safely to patients undergoing major laparotomy for colorectal carcinoma. At the same time, oral feeding can improve the return of gastrointestinal function and shorten hospital stay.

The goal of this study was to determine whether a clinical approach including early removing gastrointestinal decompression and early oral feeding is safe and shows the potential to decrease the length of postoperative stay and reduce complications after colorectostomy. Our data provide preliminary evidence that routine gastrointestinal decompression cannot reduce the pressure of gastrointestinal tract and has no obvious preventing effects upon postoperative complications. Contrary to expectations, routine gastrointestinal decompression may increase the incidence rate of fever, pulmonary infection and pharyngolaryngitis. These strategies of early removing gastrointestinal decompression and early oral feeding in the patients after colorectostomy are feasible and highly safe and are associated with reduced postoperative discomfort and can accelerate the return of bowel function and shorten postoperative stay.

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CASE REPORT

A case of colohepatic penetration by a swallowed toothbrush

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Abstract

Although foreign body ingestion is relatively common, toothbrush swallowing is rare. We report a case of a swallowed toothbrush which passed through the ileocecal valve and perforated the proximal transverse colon, then the liver. To our knowledge, this is the first case to be reported.

Key words: Toothbrush; Colohepatic penetration

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INTRODUCTION

Toothbrush ingestion is uncommon, but requires prompt medical attention. Although 80% of ingested foreign bodies pass spontaneously [1], there are no reports regarding swallowed toothbrushes passing through the pylorus [2]. Here we present an unusual case of a toothbrush swallowing which passed through the ileocecal valve and perforated the proximal transverse colon, then penetrated the liver. To our knowledge, this is the first case to be reported.

CASE REPORT

A 31 year-old man was admitted to the Surgical Department via the Emergency Room with one week history of right upper abdominal pain. He was diagnosed with schizophrenia 13 years earlier and treated at a local hospital. A physical examination revealed tenderness in the right upper quadrant and a temperature of 37 ℃. Laboratory tests showed a white blood cell count in the upper normal range, a slightly elevated C-reactive protein level (23 mg/L) and elevated aspartate aminotransferase/alanine aminotransferase levels (51/99 IU/L).

A plain abdominal radiograph showed a characteristic radiographic image of a toothbrush with parallel rows of short metallic radiodensities in the right upper quadrant (Figure 1). At that time, the patient stated he swallowed a toothbrush 1 year earlier. An abdominal computed tomography scan revealed a metallic density in the ascending colon (Figure 2A) and a low density lesion penetrating the lateral section of the liver (Figure 2B). The presumptive diagnosis was colohepatic penetration by a swallowed toothbrush and a laparotomy was performed. No abnormal ascites fluid was observed. Dense adhesions between the proximal transverse colon and the lateral section of the liver were found. When the proximal transverse colon was mobilized, the shaft of a toothbrush was observed penetrating the colon and liver (Figure 3). The toothbrush was removed and the perforated colonic opening was repaired. The extracted toothbrush was 20 cm long (Figure 4). The patient had an uneventful hospital course and was discharged eleven days after surgery.

DISCUSSION

Ingestion of a foreign body is commonly encountered in the clinic among children, adults with intellectual impairment, psychiatric illness or alcoholism, and dental prosthetic-wearing elderly subjects [1, 3]. However, toothbrush swallowing is rare, with only approximately 40 reported cases [2]. It was reported that a toothbrush shows a characteristic radiographic image with parallel rows of short metallic radiodensities due to the metallic plates that hold the bristles in place [4]. Unlike most other foreign bodies, there are no reports of swallowed toothbrushes passing spontaneously [2]. Thus, prompt intervention is required in order to avoid complications such as pressure necrosis causing gastritis, ulceration and perforation [5]. An initial extraction strategy to consider is endoscopy by a skilled technician, and the first successful performance of this procedure has been reported by Ertan *et al* [6]. If endoscopic removal is not possible and particular complications are not present, a laparoscopic approach may be an alternative to laparotomy [7].

To our knowledge, this is the first report of a swal-

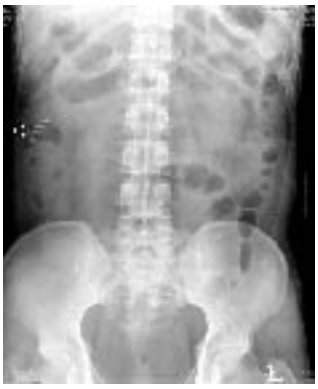

Figure 1 A plain abdominal radiograph showing a characteristic toothbrush image with parallel rows of short metallic radiodensities in the right upper quadrant (arrow).

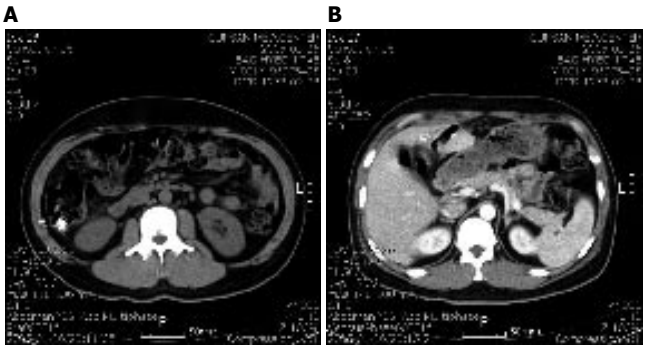

Figure 2 Abdominal computed tomography (CT) imaging. **A**: A metallic density in the ascending colon (arrow); **B**: A low density lesion penetrating the lateral section of the liver (arrow).

Figure 3 Shaft of a toothbrush penetrating the proximal transverse colon and the lateral section of the liver (arrow).

Figure 4 Extracted 20 cm toothbrush.

lowed toothbrush passing through the ileocecal valve and penetrating the colon and liver. Similar to the present case, there are reports of toothpicks penetrating the pyloroduodenal region and migrating to the liver [8]. A Medline search indicates that other similar reports involving toothbrushes are found only in the esophagus and stomach. In the present case, it is highly remarkable that a 20 cm toothbrush could pass through the pylorus and duodenal loop.

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CASE REPORT

Fulminant hepatic failure resulting from small-cell lung cancer and dramatic response of chemotherapy

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Abstract

Prompt treatment in tumor-associated encephalopathy may prolong survival. We describe a 69-year-old male patient who was presented with fulminant hepatic failure, secondary to small-cell lung carcinoma with rapidly progressing encephalopathy. Both symptoms remitted following chemotherapy, suggesting swift diagnosis and administration of chemotherapy to be effective in treatment of fulminant hepatic failure and encephalopathy.

Key words: Small-cell lung carcinoma; Fulminant hepatic failure; Chemotherapy

Kaira K, Takise A, Watanabe R, Mori M. Fulminant hepatic failure resulting from small-cell lung cancer and dramatic response of chemotherapy. *World J Gastroenterol* 2006; 12(15): 2466-2468

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INTRODUCTION

Fulminant hepatic failure (FHF) is defined as a liver failure with encephalopathy, developing within eight weeks from the onset symptoms in the absence of pre-existing liver disease. The most common causes of FHF are viral or drug-induced hepatitis[1]. Small-cell lung cancer (SCLC) manifesting as acute hepatic failure resulting from diffuse parenchymal infiltration by metastatic tumor is rare. The prognosis is poor, with death usually occurring within several days[1]. We describe herein a patient with SCLC and FHF that improved after chemotherapy.

CASE REPORT

A 69-year-old male was admitted to our hospital because of a 2-wk history of nausea, anorexia, fatigue, jaundice, and pain in the right upper quadrant of the abdomen. He had no history of blood transfusion, hepatitis, intravenous drug use, or alcohol abuse, though had an average of 2 packs of cigarettes a day for 50 years. Physical examination revealed hepatomegaly and bilateral pretibial edema, but he was alert. Laboratory analyses revealed 1760 U/L alkaline phosphatase, 6695 U/L lactate dehydrogenase (LDH), 470 U/L aspartate aminotransferase (AST), 7.6 mg/dL bilirubin, 85 μg/dL ammonia, prothrombin time level of 63%, and 680 ng/mL neuron specific enolase (NSE) levels. A computed tomography (CT) of the abdomen showed multiple lesions and massive infiltration throughout the liver parenchyma (Figure 1A). A chest roentgenogram and CT scan showed a right hilar mass with pleural effusion (Figure 1B). Bronchoscopy with transbronchial lung biopsy revealed small-cell carcinoma of the lung (Figure 2). On the seventh day of hospitalization, chemotherapy was initiated with carboplatin (area under the curve 4) administered on d 1, and 80 mg/m^2 etoposide from d 1 to 3, with cycles repeated every 3 wk.

After two cycles of chemotherapy, the patient felt symptomatically better, and a CT of the chest and abdomen revealed a marked reduction of tumor size in the primary site and liver metastasis. The laboratory analyses revealed an improvement of hepatic failure. During the 2 months of initial chemotherapy, the patient's condition deteriorated due to progression of hepatic failure (total bilirubin level, 19.5 mg/dL; LDH, 4 699 U/L; AST, 424 U/L; ammonia, 145 μg/dL; glucose, 129 mg/dL; prothrombin time, 38%), the development of ascites, and stage II encephalopathy. A CT scan of the brain revealed no evidence of cerebral metastatic disease. However, the radiological examination revealed the recurrence of SCLC. Therefore, the patient was treated with 35 mg/m^2 amrubicin from d 1 to 3, with cycles repeated every 3 wk. Though grade 4 neutropenia (NCI-CTC ver 2.0) occurred after two cycles of chemotherapy, the patient had a marked improvement of encephalopathy (stage 0), general condition, and laboratory values (LDH, 180 U/L; AST, 45 U/L; ammonia, 42 μg/dL; prothrombin time 89%; and NSE, 9.6 ng/mL). Follow-up CT imaging documented a good partial response of the liver metastases, and a complete disappearance of the right hilar mass. Then,

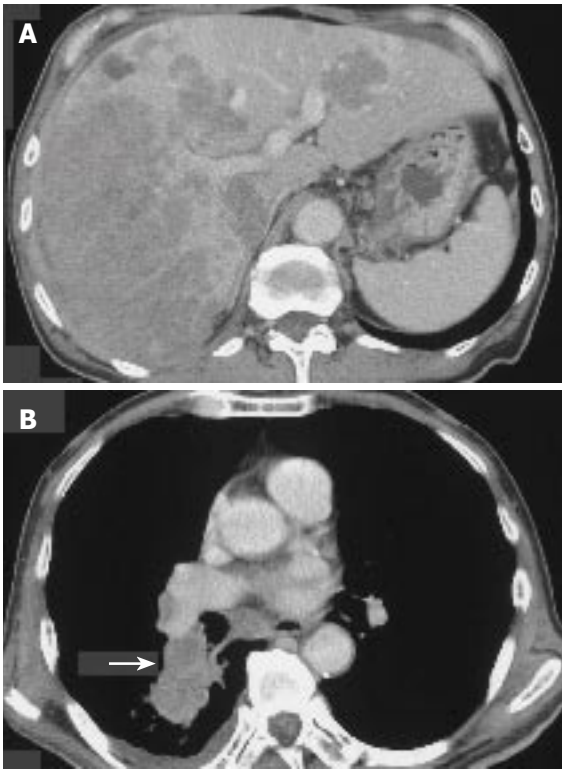

Figure 1 Computed tomography of the patient. **A**: CT of the abdomen showing multiple lesions and massively infiltrative process throughout the liver parenchyma; **B**: CT of the chest showing a right hilar mass (arrow) with pleural effusion.

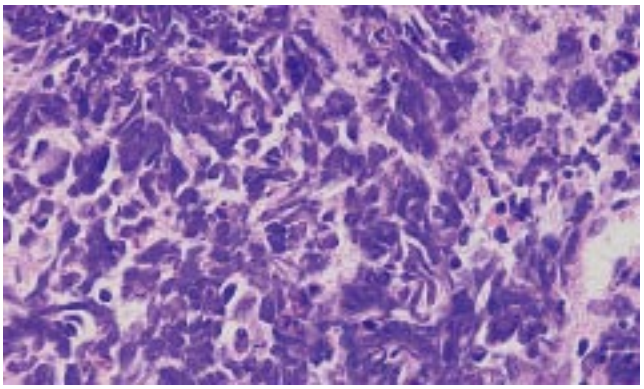

Figure 2 Histological examination of transbronchial lung biopsy specimen showing extensive infiltration small-cell carcinoma.

he was discharged from our hospital. However, the patient died of bacterial pneumonia at six months after presentation. A post-mortem was not done because of refusal of his family.

DISCUSSION

FHF secondary to metastatic SCLC is rare and results in death within days to weeks[1-3]. In literature, there are 21 previously reported cases of acute hepatic failure resulting from metastatic SCLC (Table 1). Two of the 21 reported patients presented an apparent dramatic response of severe tumor-related lactic acidosis to the initiation of chemotherapy (cyclophosphamide, doxorubicin, and vincristine)[3,4]. In most of the reported patients, however, the administration of appropriate chemotherapy was not performed, and no diagnosis was made before death. The rapid deterioration of the patient's condition may account for the difficulty of an accurate diagnosis[1]. The seven patients of acute hepatic failure with encephalopathy, even if metastatic SCLC was diagnosed before death, all died without the administration of chemotherapy because of a rapidly deteriorating course[1,5-8].

Table 1 Summary of 21 previously reported cases of acute hepatic failure due to metastatic small-cell carcinoma

References	Age (yr)	Chemotherapy	Coma	Diagnosis before death	Survival (d)
1	68	No	Yes	No	18
1	66	No	Yes	Yes	1
1	58	No	Yes	No	2
1	51	Yes	No	Yes	19
2	68	No	No	Yes	3
3	56	Yes	No	Yes	>140
4	45	Yes	No	Yes	114
5	66	No	Yes	No	3
6	85	No	Yes	No	8
7	64	No	Yes	No	6
8	46	No	Yes	No	8
12	48	No	No	No	14
12	59	No	No	No	14
12	45	No	No	No	6
13	79	No	No	No	6
14	57	No	No	No	10
14	52	No	No	No	16
15	55	Yes	No	No	5
15	57	NR[1]	NR[1]	No	NR[1]
16	46	NR[1]	NR[1]	No	9
17	65	No	No	No	20
Present case	71	Yes	Yes	Yes	>182

[1]NR: not reported.

This case presents an apparent dramatic response of tumor-related encephalopathy to the administration of chemotherapy. There was no evidence for other, more usual causes of encephalopathy. Although the combination of carboplatin and etoposide as first-line chemotherapy was effective to acute hepatic failure, it failed to exert any effect against rapid encephalopathy. However, the encephalopathy improved dramatically after the administration of amrubicin as second-line chemotherapy.

Amrubicin (SM-5887) is a totally synthetic anthracycline and a potent topoisomerase II inhibitor[9], and is one of the most active agents for SCLC. In a phase II study of amrubicin using a schedule of 45 mg/m^2 on d 1-3 every 3 wk in 33 previously untreated extensive-stage SCLC patients, an overall response rate of 76% and median survival of 11.7 mo have been observed[10]. In this case, amrubicin, as second-line chemotherapy, was effective for the improvement of encephalopathy and liver metastases. However, the efficacy of amrubicin as a salvage chemotherapy remains unknown.

Extremely high serum LDH level represents diffuse replacement of the liver parenchyma and is associated with a higher risk of developing FHF[2]. Several reports have shown correlation between serum LDH levels with survival in SCLC patients with liver metastases, and suggested an accurate relationship between elevated LDH

levels and hepatic dysfunction[1,2,7]. These findings are in agreement with the LDH levels in our patient, who was observed with elevated LDH serum levels prior to chemotherapy, then regressing to normal LDH levels after the improvement of liver metastasis.

In the reports of malignant diseases manifesting as acute hepatic failure complicated by encephalopathy, a non-Hodgkin's lymphoma patient responded to chemotherapy and survived FHF[11]. Once FHF develops secondary to diffuse liver metastases in cancer patients, prognosis is dismal and there is no effective treatment with the exception of chemotherapy. Our case, with supporting evidence from non-Hodgkin's Lymphoma[11] and 2 cases of lactic acidosis[3,4], suggests that early diagnosis and a prompt administration of appropriate chemotherapy may result in the improvement of acute hepatic failure, and thereby improving survival.

Hence, we suggest that in addition to the efficacy of amrubicin as second-line chemotherapy, early diagnosis and prompt initiation of chemotherapy may result in the improvement of FHF, and thereby improving survival.

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CASE REPORT

Bochdaleck's hernia complicating pregnancy: Case report

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Abstract

Diaphragmatic hernia complicating pregnancy is rare and results in a high mortality rate, particularly if early surgical intervention is not undertaken. We report a case in which a woman presenting at 23 wk's gestation was admitted with symptoms of respiratory failure and bowel obstruction due to incarceration of viscera through a left posterolateral defect of the diaphragm (Bochdalek's hernia). Surgery (left thoracoabdominal incision) demonstrated compression atelectasis, mediastinal shift, strangulation and gangrene of the herniated viscera which led to segmental resection of the involved portion of large intestine with re-establishment of bowel continuity by end to end anastomosis. The greater omentum was partly necrotic necessitating resection. The diaphragmatic defect was closed with interrupted sutures. Postoperative period was uncomplicated. Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed. It is concluded that symptomatic maternal diaphragmatic hernia during pregnancy is a surgical emergency and requires a high index of suspicion.

Key words: Pregnancy; Diaphragmatic hernia; Incarceration

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INTRODUCTION

Diaphragmatic hernia complicating pregnancy is rare and is associated with a poor or complicated outcome, particularly if early surgical intervention is not undertaken[1]. Such hernias usually involve the left diaphragm through a congenital defect or a previous traumatic rupture. The main life-threatening complications described, include respiratory distress, visceral obstruction, strangulation and gangrene of the herniated viscera, visceral perforation (spontaneous or thoracentesis-induced) and maternal death[2-5].

A case of a patient with left-sided congenital diaphragmatic hernia during pregnancy is described in this report.

CASE REPORT

A 31-year-old woman in her first pregnancy (23 wk's gestation) was admitted to our hospital with a 10-day history of dyspnoea, nausea, persistent vomiting and intermittent sharp epigastric and substernal pain leading to a 3 kg weight loss. On physical examination the patient was afebrile, hypotensive with tachycardia (125 beats/min) and dyspnoea (32 breaths/min). There was distention of the neck veins fullness of the supraclavicular fossa. Chest auscultation demonstrated absence of breath sounds in the left hemithorax. Heart sounds were heard to the right of the sternum. Her abdomen was soft and moderately tender over epigastrium and left hypochondrium with audible bowel sounds. Laboratory findings were remarkable only for a white blood count of 13 800/mL (with 88% neutrophils). Electrocardiogram indicated sinus tachycardia and a T-wave flattening. A nasogastric tube was placed and returned 800 mL bilious fluid.

A chest X-ray suggested diaphragmatic hernia because of air bubbles, air-fluid level and non-homogeneous opacity in the left hemithorax (Figure 1). A right mediastinal shift and loss of the sharp left hemidiaphragm line separating the abdomen from thorax, were also noted.

A chest ultrasound examination demonstrated bowel loops in the left pleural cavity and slight pericardial and pleural effusions and confirmed definitely the diagnosis of diaphragmatic hernia. It is generally considered that the hernia could become manifest on a basis of anatomical weakness possibly evoked by a trauma or increasing abdominal pressure, however, the patient denied any previous trauma.

Due to her unstable condition and because of concerns for ischemia of the herniated viscera, the patient was rushed to surgery. On exploration through a left thoracoabdominal incision, a large segment of right and transverse colon, the greater omentum and the stomach were identified in the left hemithorax. Compression atelectasis, mediastinal shift, strangulation and gangrene of large part of the herniated colon were also noted (Figure 2). The

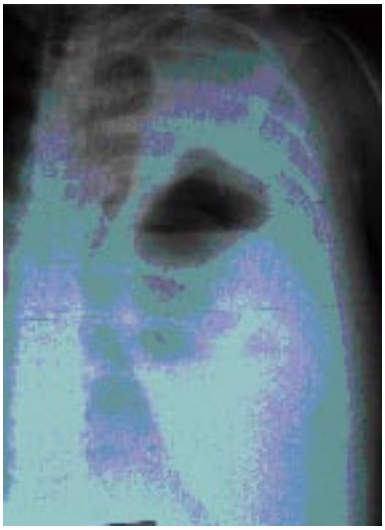

Figure 1 Chest radiograph: Herniation of abdominal viscera in the left hemithorax.

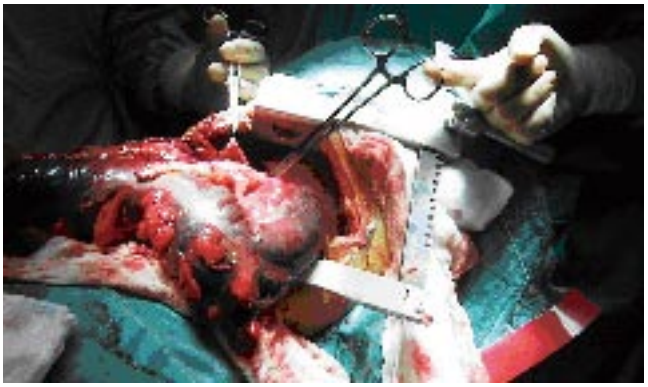

Figure 2 Gangrene of a large part of the herniated transverse colon.

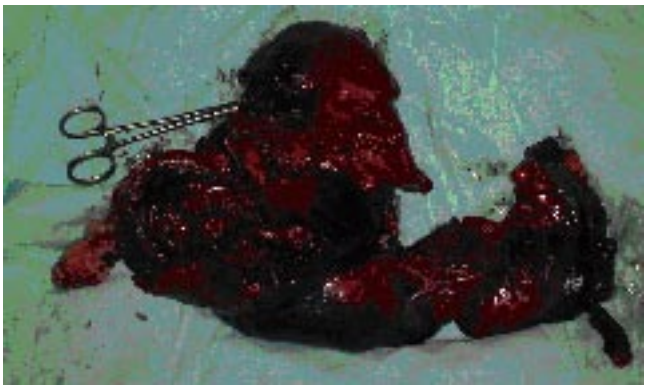

Figure 3 Large part of transverse colon resected due to strangulation and gangrene.

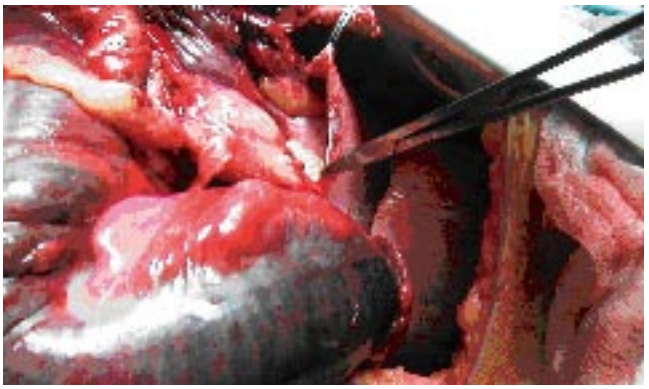

Figure 4 Bochdaleck's defect in the left hemidiaphragm.

viscera had protruded upward through a 4 cm × 7 cm posterolateral foramen of Bochdaleck diaphragmatic defect (Figure 3). The greater omentum seemed to be ischemic. A segmental resection of the involved portion of large intestine with re-establishment of bowel continuity by an end to end anastomosis was mandatory (Figure 4). Stomach was reduced in the abdominal cavity with no evidence of ischemic damage. The diaphragmatic defect was repaired with interrupted sutures. Manipulation of the uterus was avoided during surgery. Fetal well-being was confirmed throughout the procedure by continuous fetal heart rate monitoring.

After surgery, the patient recovered rapidly and her complaints ceased immediately. The postoperative course was uneventful and fetal and uterine activities were monitored daily. The patient was discharged home on postoperative day 10 after she tolerated a regular diet.

Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed.

DISCUSSION

The diaphragm develops during the first 8 wk of gestation. In the eighth week this developing diaphragm leaves two channels located posterolaterally (Bochdaleck) and anteriorly (Morgagni) between the thorax and the abdomen. These two channels are closed by a two-layer membrane derived from pleura and peritoneum. When the posterolateral pleuroperitoneal channels fail to close, these are called foramina of Bochdaleck. Usually, it is rare for a defect like this to remain undetected in childhood through to adult life[6]. When the defect becomes manifest during pregnancy life-threatening events may affect both the mother and fetus. This is due to incarceration or strangulation of intraabdominal structures.

There are various symptoms in patients with congenital diaphragmatic hernia. Most of them are due to the effects of abdominal viscera within the pleural cavity and the commonest are chest pain and dyspnoea. Diminished breath sounds on the ipsilateral side are the most common physical finding. There may be signs of high or low mechanical ileus depending on which part of the gastrointestinal tract is herniated. In our patient, intraabdominal viscera herniated into the chest and caused dyspnea due to striking collapse of the left lung. Tachycardia, hypotension and distention of neck veins were the result of mediastinal shift and diminished venous return. Persistent vomitus was a sign of obstruction. However heartburn, nausea, vomiting and malaise are common symptoms during pregnancy. The failure of antacids, antispasmodics and dietary changes to relieve the symptoms especially in women with advanced pregnancy should lead the physician to suspect gastrointestinal pathology[7].

The diagnosis of a congenital diaphragmatic hernia during pregnancy requires a high index of suspicion. As in our case, the key to diagnosis is the chest radiograph. Thoracic ultrasonography, computed tomography scans and magnetic resonance imaging are possible auxilliary diagnostic methods. Pleural effusion or pneumothorax may be mimicked, leading to inappropriate thoracentesis or tube thoracostomy and inadvertent perforation of the herniated viscera[8].

The management of a pregnant patient with symptomatic diaphragmatic hernia is challenging. Traditionally, immediate repair is undertaken if symptoms arise, because further delay might be fatal both to the mother and fetus[5,6] . For asymptomatic patients Kurzel and Naunheim recommended cesarean delivery after fetal lung maturity is documented, with simultaneous hernia repair[6]. They opposed vaginal delivery under any circumstance because of the increased risk of incarceration with subsequent strangulation when the patient is bearing down. They based their recommendation on 17 cases reported in the English literature. In that case series the maternal and fetal morbidity was as high as 55% and 27% respectively, when vaginal delivery was attempted before the diaphragmatic hernia was repaired[6]. Genc *et al* suggested that gastric decompression might improve the clinical condition of the pregnant patient with a diaphragmatic hernia who presents with symptoms and signs of obstruction[9]. Such an improvement can allow surgery to be delayed until the patient is transferred to a tertiary care center or until antenatal corticosteroids are administered. Given this fact, Genc *et al* suggested observation and proposed immediate surgery whenever there is any suspicion of visceral incarceration. In our case, symptoms of cardiorespiratory failure and bowel obstruction led us to urgent repair because further delay could be fatal for both mother and fetus. Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed.

The incidence of asymptomatic Bochdaleck's hernia in the adult population was reported to be at least 0.17%, with a female-to-male ratio of 17:5[10]. Despite such a high incidence, the number of pregnancies complicated by unrecognized congenital diaphragmatic hernia is extremely small - 35 cases, including this one, have been reported since 1928[5,9].

In conclusion, the diagnosis of congenital diaphragmatic hernia complicating pregnancy requires a high index of suspicion. Symptoms like abdominal pain, nausea, vomiting and dyspnoea should be investigated adequately. Signs of bowel obstruction and respiratory compromise are indications for a surgical emergency.

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Meetings

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American College of Gastroenterology Annual Scientific
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World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
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International Conference on Surgical Infections, ICSI2006
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European Society of Clinical Microbiology and Infectious Diseases
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5th International Congress of The African Middle East Association of Gastroenterology
24–26 February 2006
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www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
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Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
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laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26–28 January 2006
San Francisco - CA
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

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Seatlle - Washington
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71st ACG Annual Scientific and Postgraduate Course
20–25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual – The Liver Meeting™
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Boston, MA
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New York Society for Gastrointestinal Endoscopy
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EVENTS AND MEETINGS IN 2007

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Barcelona
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4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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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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 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_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_r18 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 $\cdot L^{-1}$	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	μmol	
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	A_{260} nm	*A* should be in italic.
	^{b}P<0.05	^{a}P<0.05	In Table, no note is needed if there is no significance instatistics: ^{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.
45	**F*=9.87, $^{\S}F$=25.9, $^{\#}F$=67.4	^{1}F=9.87,^{2}F=25.9, ^{3}F=67.4	Notices in or under a table
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/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	