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Editorials

Molecular mechanisms of *H. pylori* associated gastric carcinogenesis

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H. pylori infects half of the world population and the prevalence varies widely in different parts of the world with average rates of 40%-50% in western countries, rising to more than 90% in the developing world[1,2]. Compelling evidence from epidemiological and histopathological studies has linked *H. pylori* infection to the subsequent development of gastric carcinogenesis[3]. Furthermore, Watanabe and colleagues recently induced gastric adenocarcinoma in 37% of orally infected Mongolian gerbils, which were preceded with a series of premalignant changes in gastric mucosa of these gerbils[4]. However , in spite of the established causal relationship between *H. pylori* infection and gastric carcinogenesis, the underlying mechanisms remain unknown.

Disturbances in cell turnover in the gastrointestinal tract are believed to predispose to cancer development, and until recently, these changes were considered to be a marker of increased cancer risk[5]. It is clear that this organism is the main cause of chronic gastritis and capable of modifying epithelial cell turnover within gastric glands and in culture gastric epithelial cells, by influencing the balance between cell proliferation and apoptosis[6-9]. We and others have studied the effect of *H. pylori* infection on gastric epithelial cell turnover and found that patients infected with *H. pylori* had significantly higher proliferation rates as compared with uninfected controls[6,8-12]. The density of *H. pylori* may be one of the important determinants as we found that *H. pylori* at low inocula, stimulates cell proliferation, but at higher inocula (bacterium to cell ratio >100), it causes a time- and concentration-dependent reduction of cell number and a marked increase in apoptosis and cell cycle arrest at G1 phase[8]. Antioxidant vitamins C, E and β-carotene affect cell growth in various human cells directly or through their a ntioxidant properties[13,14]. Our *in vitro* studies showed that the a bove vitamins can also significantly inhibit gastric cancer cell proliferation and induce apoptosis[15].Investigations on gastric vitamins in patients with and without *H. pylori* infection suggest that *H. pylori* infection affects the concentrations of vitamin C, E and β-carotene in the stomach and the $CagA^+$ strains have an even greater ability to reduce gastr ic juice vitamin C levels[16,17].

Although many factors may be related to *H. pylori* associated gastric carcinogenesis, the underlying molecular mechanisms are still unknown. However, many mediators and signal transduction pathways are involved in the regulation of gastric epithelial cell homeostasis, some of which may determine the final outcome of *H. pylori* infection. Understanding the molecular basis of *H. pylori* associated gastric carcinogenesis is important for determining prognosis, prevention and treatment of *H. pylori* infection. This review examines the possible mo lecular mechanisms responsible for *H. pylori* associated gastric carcinogenes is.

BACTERIAL VIRULENCE FACTORS

Although many factors contribute to *H. pylori* virulence, few have been directly related to gastric carcinogenesis[18]. Most strains of *H. pylori* from patients with intestinal-type gastric cancer or with atrophic gastritis are type I and secrete vacuolating cytotoxin (VacA) and carry CagA, a gene that encodes an immunodominant protein of unknown function, whereas many of the strains from asymptomatically infected persons lack this gene[19-21]. It has been shown that patients harbouring $CagA^+$ strains have significantly higher gastric epithelial proliferation rates than patients infected with $CagA^-$ strains, but the apoptotic index in patients infected with $CagA^+$ strains are lower than in patients infected with $CagA^-$ strains. Increased cell proliferation in the absence of a corresponding increase in apoptosis may explain the increased gastric cancer risk associated with infection by $CagA^+$ strains[10]. Although there is no direct evidence suggesting that the CagA or VacA

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protein is carcinogenic, the enhanced inflammation and marked reduction of gastric juice vitamin C levels in $CagA^+$ strain infected patients may ply a role in *H. pylori* associated carcinogenesis[16].

H. pylori expresses a powerful urease enzyme, which catalyses the conversion of urea to ammonia. Individuals with *H. pylori* infection have higher ammoni a concentrations in gastric juice than uninfected controls[22]. A series of studies have demonstrated that concentrations of ammonia comparable to those found in gastric juice of infected individuals can cause gastric atrophy in rats, increase epithelial cell proliferation, and act as a promoter in the methyl- *N'* nitro-*N*-nitrosoguanidine (MNNG) rat model of gastric cancer[23-28].

Furthermore, *H. pylori* phospholipases, proteases and oxidases have been shown to cause degradation of many molecules, such as phospholipids in bio-membranes, transforming growth factor-β (TGF-β) and vitamin C, which are important in preventing carcinogenesis[16,29-31].

OXIDATIVE DNA DAMAGE AND p53

Mutation of the p53 tumor suppressor gene is the most common alteration found in a variety of human tumor cells and is considered to be one of the steps leading to the neoplastic state. The importance of p53 protein may be due to its effect on cell cycle progression, including cell proliferation and apoptosis, in response to cell DNA damage[32]. The activated p53 protein can affect the expression of a number of genes, including the cyclin-dependent kinase inhibitor (CKI) p21, which regulates G1 cell cycle check point and bax, a gene involved in apoptosis. Therefore, the protein can either prevent the cell from entering the S phase until the DNA damage is repaired and/or can turn on the apoptotic pathways to destroy an abnormal cell[33].

The p53 gene abnormalities in gastric cancer are usually point mutations or allelic deletions leading to over-expression of the protein, loss of p53 function and with resulting defects in the protective pathways of cell cycle arrest and apoptosis. Furthermore, the increased p53 expression and gene mutations have also been reported in gastric premalignant mucosa, such as dysplasia, atrophy or even the mucosa without obvious abnormality, suggesting that p53 function is affected from the early stage of gastric carcinogenesis[34].

The role of p53 in *H. pylori* associated carcinogenesis is still unclear, but some evidence suggests that it may be protective in this process. In p53 knockout mice, atrophic gastritis developed in 2 of 4 animals infected with *H. felis* within 3 months including one which developed moderate dysplasia. In contrast, these changes were not seen in any of the control animals, suggesting that lack of functional p53 accelerated carcinogenesis in experimental *Helicobacter* infection[35]. Data from Fox and colleagues further support the protective role of p53. They examined the effect of infection with *H. felis* in heterozygous mice deficient in one p53 allele[36]. One year after infection, the wild-type and p53 heterozygous mice both showed severe adenomatous and cystic hyperplasia of the surface foveolar epithelium. However, infected p53 heterozygous mice had a higher proliferative index than the infected wild-type mice.

Whether *H. pylori* and its associated inflammation induces p53 mutations or affects the activity of the protein is not clear, but p53 function may be defective at an early stage in *H. pylori* associated gastric carcinogenesis. Some studies have reported increased expression of p53 protein in gastric mucosa infected with *H. pylori*. However, the enhanced p53 expression failed to have any effect on gastric epithelial cell proliferation or apoptosis, and there appeared to be a positive relationship between the accelerated cell turnover and p53 over-expression. The accumulation of p53 was also not associated with expression of the CDI p21, a down-stream effector of p53[37,38]. We have recently in itiated a study investigating the role of p53 in *H. pylori* infected gastric cell lines and found that *H. pylori* associated apoptosis is independent to p53 status of gastric cells[39]. These findings suggest that p53 function is defective in *H. pylori* infected mucosa. There are several mechanisms wh ich may lead to the loss of p53 function. Firstly, recent studies suggest that p53 mediated apoptosis is suppressed by signals from growth factors, such as inte rleukin-6 (IL-6), interferon-γ (IFN-γ) and protein kinase C, which are shown to be up-regulated by *H. pylori* infection[40-42]. Secondly, it is likely that *H. pylori* and its associated inflammatory responses cause p53 gene mutation. *H. pylori* infection induces increased production of reactive oxygen metabolites (ROMs) by persistent inflammatory cell infiltration in gastric mucosa[43-45]. Many studies have indicated that ROMs can directly interact with genomic DNA and cause damage in specific genes that control cell growth and differentiation[46-48]. Furthermore, it has been reported that intact *H. pylori*, as well as isolated cellular components, stimulate nitric oxide (NO) synthesis[49-51]. High concentrations of NO induce wild-type p53 protein accumulation[52,53], and the NO-related deamination of DNA has been reported to cause GC-AT transitions, which are frequently found in p53 mutations in gastric cancer[23,54]. In addition, *H. pylori* possesses several proteases which may

directly affect p53 activity though there is no direct experimental evidence of a relationship between these proteases and p53 protein[55].

BCL-2 AND OTHER APOPTOSIS RELATED MOLECULES

Bcl-2 protein is a part of a large group of proteins encoded by specific genes belonging to the bcl-2 family, which are known to play an important role in reg ulation of apoptosis. Some of these proteins (bcl-2 and bcl-xL) support survi val, whereas others (bax, bad, bcl-x5) are apoptosis inducers[56]. Over-expression of bax and bak proteins encoded by the two pro-apoptotic members of the *bcl-2* gene family has been associated with *H. pylori* infection and to induce apoptosis in the AGS gastric epithelial cell line and in gastric mucosal biopsi es from patients colonized by *H. pylori*[57]. In contrast, the expression of bcl-2 protein was not affected or even suppressed by this organism[37]. However, over-expression of bcl-2 with abnormal distribution of apop totic cells along the glands, which are usually found at the extremities of normal gastric glands, has been described in both intestinal metaplasia and gastric dysplasia, which occur as a result of long-term *H. pylori* infection[58,59].

Several other molecules may also play a role in *H. pylori* associated apoptosis. Treatment of gastric cells with TNF-α and IFN-γ markedly pot entiate *H. pylori* induced apoptosis[60]. Rudi *et al* have recently reported that *H. pylori* up-regulates the expression of the CD95 (APO-1/FAS) and CD95 ligand (CD95L), which are involved in initiating apoptosis. In fact, the enhanced CD95 expression observed in this study was associated with incre ased rates of apoptosis in gastric epithelial cell lines and in gastric mucosa[61]. The importance of this study is not only the demonstration of the in volvement of CD95 mediated apoptotic pathway in *H. pylori* associated apoptos is, but also the expression of CD95L mRNA on surface epithelium and pyloric gland cells of *H. pylori* infected mucosa at levels comparable to those found on lamina propria lymphocytes. CD95L is normally expressed by activated T cells and recently it has been found that many tumors, including gastric adenocarcinomas express CD95L[62]. CD95L can induce apoptosis of activated immunocytes and is thought to contribute to tumor immune escape[62]. The enhanced expression of CD95L mRNA in *H. pylori* infected gastric mucosa may suppress normal immune responses by inducing immunocyte apoptosis, which may further potentia te genetic instability due to the defect in the DNA damage-p53 mediated protect ive pathway.

TELOMERASE ACTIVITY

Activity of telomerase, which synthesizes the telomeric DNA to replaces the loss that occurs at each cell division, is suppressed in most normal human somatic cells but induced in most human cancers. Normal human cells progressively lose telomere sequences due to the lack of telomerase activity. In contrast, most immortalized cell lines and malignant human tumors appear to maintain constant telomere length via the activation of telomerase[63,64]. Reactivation of telomerase is thought to be an important step in carcinogenesis.

Expression of human telomerase RNA (hTR) and telomerase activity have been studied in gastric cancer and corresponding non-cancerous mucosa[65]. Telome rase activity was detected in 23 of 26 carcinoma tissues. Although all tumor specimens and non-cancerous mucosa expressed various levels of hTR, 21 cases expressed hTR at a higher level in the tumor than that in the adjacent normal mucosa. Nine of 26 non-cancerous mucosa showed telomerase activity and all of them contained intestinal metaplasia. The incidence of telomerase-positive mucosa in grade II intestinal metaplasia was significantly higher than that in mucosa with grade I intestinal metaplasia or without intestinal metaplasia, whereas hTR over-expression was found both in mucosa with and without intestinal metaplasia regardl ess to their grades. The level of hTR expression and telomerase positivity was shown to increase in parallel with the degree of *H. pylori* infection. These results suggest that *H. pylori* infection may be a strong trigger for hTR over - expression in intestinal metaplasia, and this may lead to telomerase reactivation[65,66].

Recently, Chin and colleagues examined the interaction between telomere dysfunction and p53 in cells and organs of telomerase-deficient mice. Coincident with severe telomere shortening and associated genomic instability, p53 is activated, leading to growth arrest and/or apoptosis. Deletion of p53 significantly attenuated the adverse effects of telomere dysfunction, but only during the earliest stages of genetic crisis. Correspondingly, the loss of telomere function and p53 deficiency cooperated to initiate the transformation process[67]. These findings suggest that p53 deficiency and telomerase reactivation in *H. pylori* infected mucosa may play an important role in *H. pylori* associated gastric carcinogenesis.

HOST GENETIC FACTORS

Beales *et al* reported that the frequency of DQ 5, one of the D-related human leukocyte antigen (HLA) molecules, was significantly higher in individuals with gastric atrophy or metaplasia than in those without gastric atrophy or intestinal

metaplasia and in uninfected individuals[68]. Azuma and colleagues have studied the relationship between *H. pylori* infection and the genotyping of another D-type HLA molecule, DQA1 in 82 gastric adenocarcinoma patients and 167 unrelated controls. They found that the allele frequency of DQA1 was significantly lower in *H. pylori* positive atrophic gastritis group than in *H. pylori* positive superficial gastritis and normal control groups. In addition, the al lele frequency of DQA1 also was significantly lower in *H. pylori* positive intestinal type gastric adenocarcinoma group than in normal control, *H. pylori* positive superficial gastritis, and diffuse type gastric adenocarcinoma groups[69].

The importance of host genetic factors in determining the outcome of *H. pylori* infection was further demonstrated by Sakagami and colleagues. They assessed *H. pylori* infection in different strains of mice and found that the level of bacterial colonisation, the severity of gastritis and the development of gastric atrophy varied within these mice. For example, in infected SJL, C3H/He, DBA/2, and C57BL/6 mice, moderate to severe chronic active gastritis was observed only in the body of the stomach, which increased in severity over time with specialised cells in the body glands being replaced. As the severity of this damage in the body increased and atrophic changes were seen, the level of bacterial colonisation of the antrum decreased. In contrast, in BALB/c and CBA mice, there was only mild gastritis in the antrum, no remarkable changes were detected in the gastric body mucosa, and no atrophy was seen over time. In both these strains of mice, heavy bacterial colonisation was seen, which tended to increase over the period of the experiment[70]. These findings suggest that the host genetic factors are important in *H. pylori* associated gastric carcinogenesis.

SUMMARY

Many molecules are involved in *H. pylori* associated gastric carcinogenesis. However, how the organism and its associated inflammation, interact with these molecules in gastric mucosa to induce carcinogenesis is still unknown. Over many years, *H. pylori* infected mucosa may experience sequential exposure to "damage-regeneration" (Figure 1). Following the long-standing repeated dama ge-regenerate cycle, gastric atrophy and intestinal metaplasia gradually develop, which finally results in adenocarcinoma. During this process, the loss of p53 function may play an important role. As mentioned above, *H. pylori* infection may cause deficiency of p53 function and subsequently, this may not only lead to defects in the DNA damage-p53 mediated protective pathway, but the mutated p53 protei n may also provide a possible selective advantage for tumor cell proliferation by attenuating apoptosis[71]. Furthermore, *H. pylori* infection has be en shown to induce the reactivation of telomerase and to cause telomere dysfunction, which may cooperate with p53 deficiency to accelerate carcinogenesis[67]. In early stages of *H. pylori* infection, CD95 and bax mediated apoptos is may play an important role in eliminating damaged DNA or gene mutated cells, thereby maintaining genetic stability. However, *H. pylori* infection induces CD95L expression, which may suppress host immune responses by causing immunocyte apoptosis. Therefore, as shown in Figure 2, the loss of p53 function, reactivation of telomerase activity, inhibition of host immune responses, together with host genetic factors, may play important roles in the development of *H. pylori* associated carcinogenesis.

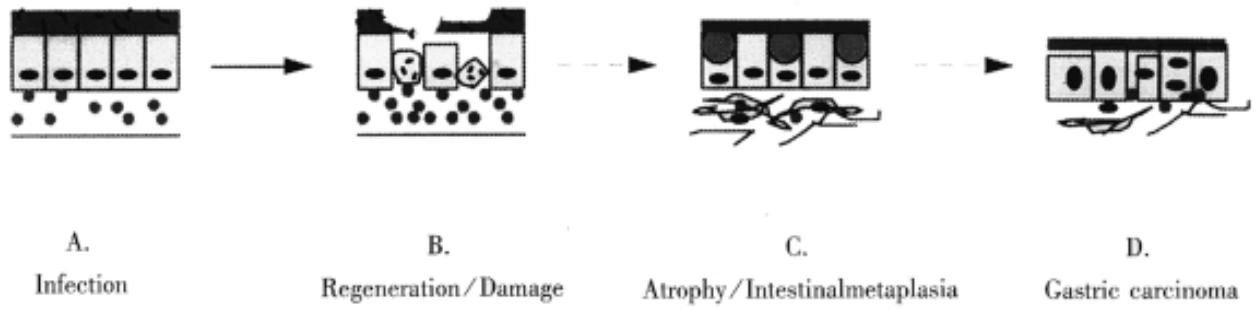

Figure1 Long-term *H. pylori* infection may indu ce a repeated "damage-regeneration" process which gradually leads to gastric atrophy, intestinal metaplasia and finally carcinoma. A. *H. pylori* infected gastric mucosa with inflammatory cell infiltration. B. Gastric epithelial cells are damaged, become apoptotic and then are regenerated, with enhanced inflammato ry cell infiltration and disturbance of mucus layer. C. Atrophy and intestinal metaplasia develop; fibrosis and thinning of the lamina propria; finally *H. pylori* is lost due to inhospitable mucosa. D. Gastric carcinoma is induced.

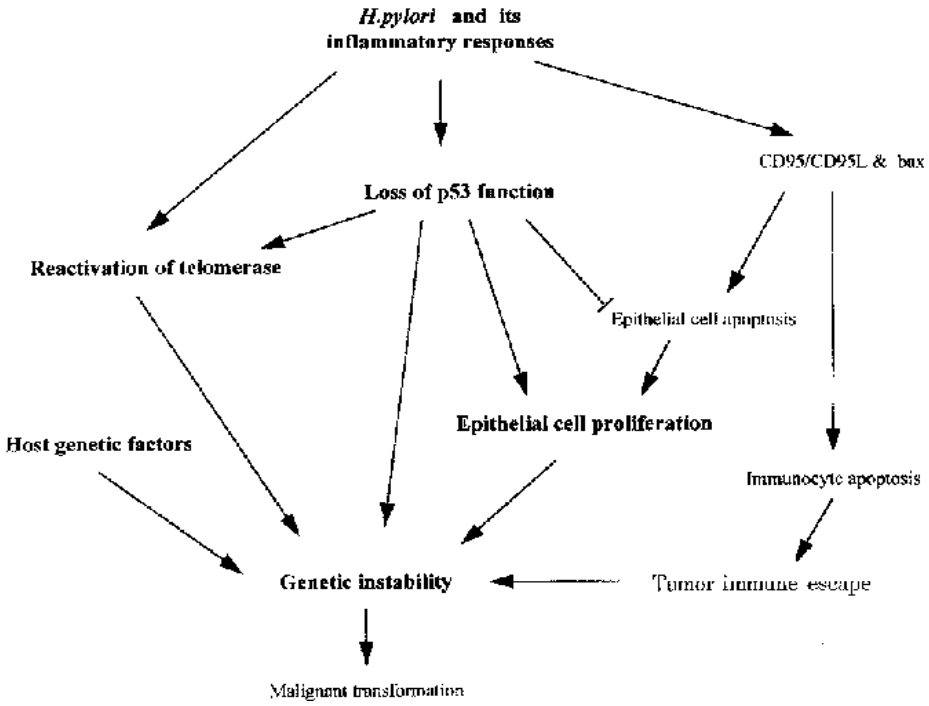

Figure2 Possible molecular mechanisms of *H. pylor i* associated carcinogenesis. *H. pylori* and its associated inflammatory re sponses cause reactivation of telomerase, the loss of p53 function and also indu ce CD95 and bax mediated apoptosis. CD95L produced by gastric epithelial cells m ay also cause intra-mucosal immunocyte apoptosis, which could facilitate tumor immune escape. However, mutated p53 may attenuate epithelial cell apoptosis, pro viding a possible selective advantage for tumor cell proliferation. Furthermore , p53 deficiency may cooperate with telomere dysfunction to accelerate carcinoge nesis. All of these changes, together with host genetic factors may play importa nt roles in the development of *H. pylori* associated carcinogenesis.

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Diagnosis and treatment of gastroesophageal reflux disease in infants and children

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Gastroesophageal reflux (GER) is a physiologic phenomenon occurring occasional ly in every human being, especially during the postprandial period. Regurgitatio n occurs daily in almost 70% of 4-month-old infants and about 25% of their par ents do consider regurgitation as “a problem” [1,2]. Indeed, it seems against logic that the normal function of the stomach would reflux ingested material back into the esophagus. Whether all infants presenting with regurgitation need drug treatment is a controversial question.

DEFINITION

GER is best defined as the involuntary passage of gastric contents into the esophagus. The origin of the gastric contents can vary from saliva, ingested food and drinks, gastric, pancreatic to biliary secretions. Vomiting is used as a synonym for emesis, and means that the refluxed material comes out of the mouth “with a certain degree of strength” or “more or less vigorously”, usually involuntary and with sensation of nausea. Regurgitation is used if the reflux dribbles effortlessly into or out of the mouth, and is mostly restricted to infancy (from birth to 12 months) [2,3]. Vomiting can be regarded as the top of the iceberg in its relation to the incidence of GER-episodes.

CLINICAL PRESENTATION

Symptoms of reflux may be observed in normal individuals, but in those cases they are only found incidentally, and they occur more often and are more severe in pathological situations. The usual manifestations and unusual presentations of GER(-

disease) are listed in Table 1 [3]. Infants with a Roviralta Astoul syndrome have pyloric stenosis associated with hiatal hernia.

Emesis and regurgitation are the most common symptoms of “primary” GER-diseas e but they are also a manifestation of many other diseases [2,3]. Such “ secondary” GER-disease can be caused by infections (e.g. urinary tract inf ection, gastroenteritis, etc.), metabolic disorders and especially food allergy [2,4]. On clinical grounds, “secondary” reflux may be difficult to sep arate from “primary” reflux. “Secondary” reflux is the result of a stimulati on of the vomiting center in the dorsolateral reticular formation by all kinds o f efferent and afferent impulses (visual stimuli, the olfactory epithelium, laby rinths, pharynx, gastrointestinal and urinary tracts, testes, etc.). “Secondary ” GER is not further discussed in this paper. It is obvious that treatment of “primary” GER-disease should focus on motility and/or acid suppression, and that therapeutic management of “secondary” GER should focus on the etiolo gic phenomenon.

PATIENT GROUPS

The following approach is a generalization that, like all generalizations, may need to be modified for an individual patient [3]. First, interest is focused on uncomplicated GER, mostly restricted to regurgitating infants. In a second paragraph, a proposal is made for optimal management in patients with complicated GER disease (symptoms suggestive of esophagitis). There is a continuum betw een normal infants with regurgitation and GER and those with severe GER which leads to disability, discomfort or impairment of function. An approach is proposed for the management of patients with atypical presentations of GER.

Group 1. Uncomplicated reflux: regurgitation

Regurgitation may occur in children who are normal and do not have complaints of GER-disease such as nutritional deficits, esophagitis, blood loss, structures, apnea or airway manifestations. There is no difference in the incidence of regu rgitation in breast-fed and formula-fed infants [5]. But, infants with u ncomplicated regurgitation are frequently perceived by their parents as having a problem, and their parents often seek medical attention. The approach of the in fants presenting with “excessive” regurgitation and of their parents has to be well balanced, and cannot be subject to overconcern or disregard. This group of patients are mostly restricted to infants younger than 6 months, or at the most 12 months [1,3,5]. A careful history, observation of feeding, and physic al examination of the infant are mandatory. Although the following statement has not been thoroughly validated because randomization is not possible (only anxio us parents seek medical help), it is rather unlikely that regurgitation will res ult in severe GER-disease. The effect of parental reassurance is suggested by m any placebo-controlled studies showing a similar efficacy of placebo and the tested intervention [6,7]. If simple reassurance fails, dietary intervention is recommended, including restriction of the volume in clearly overfed babies, and change to a thickened “anti-regurgitation” formula [5-7]. Larger food volumes and high osmolality increase the number of transient lower esophageal s phincter (LES) relaxations and drifts to almost undetectable levels of LES-pres sure [8].Both are well known pathophysiologic mechanisms provoking GER in infants, which might also explain why feed thickeners sometimes aggravate their symptoms. The thickening of the formula, with starch (e.g. from rice, potato, etc.) or non-nutritive thickeners (bean gum), decreases the frequency and volume of regurgitation [5-7,9] (Table 2). Some of these “anti-regurgitation” fo rmulae are casein-predominant (casein/whey 80/20%) to optimize the curd for mation, while others contain 100% whey (hydrolysate) enhancing gastric emptying. However, the effect of these formulae on GER-parameters, when measured with pH monitoring

or scintigraphy are not convincing: most studies show that reflux pa rameters can improve, remain unchanged or worsen in approximately one third of i nfants for each possibility [6,7,10]. In other words, “anti regurgitati on” formulae do what they claim to do: they reduce regurgitation [5-7] but they do not influence (acid) GER. Thickened formula also increases the duration of sle ep [5,6]. Therefore, anti-regurgitation formula should be considered as the first step in medical treatment, and should only be available on prescription [3,5-7]. Anti-regurgitation formula and/or dietary intervention in gen eral should be nutritionally safe [34]. However, regurgitation may be par t of the spectrum of symptom(s) of GER-disease, necessitating an effective inte rvention to decrease the number and intensity of the GER-episodes. In this situation, an intervention that is limited to alleviate the presenting manifestation (regurgitation) will not suffice. Differentiation between regurgitation and (pathologic) vomiting can be difficult on clinical grounds, since there is a continuum between both conditions [5]. It is not always obvious in this patient group whether the parental complaints relate to physiological regurgitation or whether they suggest GER-disease. In practice, feed thickeners or special formula ca n not be given to breast-fed infants. Therefore, if the infant is breast-fed a nd/or in case of GER-disease, drug treatment with prokinetics should be consi dered prior to diagnostic procedures.

It seems reasonable to add medication such as prokinetics to the treatment of cases that are refractory to dietary intervention. They reduce regurgitation via their effects on the LES pressure and motility, esophageal peristalsis and gastric emptying [11]. For this reason, they interact with the pathophysiologic mechanisms of regurgitations in infants, which are related to immaturity of the gastroesophageal motor function [12]. A link between cisapride and increased salivary secretion has been demonstrated [13]. This indicates that, in combination with increased peristalsis and hence esophageal clearance, cisapride therapy may protect the esophagus via salivary components, such as bicarbonate and non-bicarbonate buffers, thus facilitating symptomatic relief and healin g of the esophagus.Metoclopramide and domperidone have anti-emetic properties due to their dopamine-receptor blocking activity, whereas cisapride is a prokinet ic acting through indirect release of acetylcholine in the myenteric plexus [11]. Although all three agents have been shown to reduce regurgitation in i nfants [6,7], data for cisapride are more convincing (Tables 3, 4). When compared to metoclopramide, cisapride appears to be more effective in reducing p H-metric [14], has a faster onset of action [15], and is better t olerated [15]. Cisapride has also been shown to heal oesophagitis [16]. Domperidone has been reported to be as effective as metoclopramide [17] (less effective than cisapride). Extrapyramidal reactions and increased pro lactine levels are effects related to the dopamine-receptor blocking activity o f these drugs. In case of cisapride, which is devoid of dopamine- blocking prope rties at therapeutic doses, the most common adverse effects are transient diarrhea and colic (in about 2%) [11,18]. The isolated reports of more serious adverse reactions, i.e., side-effects on the central nervous system, including extr apyramidal reactions and seizures (in epileptic patients), cholestasis (in extre me prematures) and cardiac interactions. Indeed, cisapride, which is metabolized by the cytochrome P450 3A4, has the potential to prolong the QT-interval [18]. However, an extensive review of the literature resulted in reassuring sa fety consensus statements [18].To date, serious cardiac adverse reactions have not been reported in patients treated with a dosage within the recommended regimen (0.8mg/kg daily, max. 40mg/day) and in the absence of a dditional risk factors (Table 4). The association of cisapride with systemic or oral azole antifungals and

with macrolides is contraindicated. Both azole-antif ungals and macrolides interact with the cytochrome P450 3A4, resulting in elevated cisapride plasma levels. In view of its mode of action, efficacy and safety, as well as its lower or equal cost when compared to other therapeutic agents for GER, cisapride is recommended when dietary treatment fails or in regurgitating breast-fed infan ts, if therapy is indicated. It merits consideration that prokinetics stimulate a physiologic activity (peristalsis), while acid-suppressive medication inhibit s a physiologic secretion.

Table 1 Symptoms of GER(-disease)

Usual manifestations Specific manifestations	Symptoms possibly related to complications of GER*
Regurgitation Nausea Vomiting	Symptoms related to anaemia (iron deficiency anaemia) Haematemesis and melaena Dysphagia (as a symptom of oesophagitis or due to stricture formation) Weight loss and/or failure to thrive Epigastric or retrosternal pain “Non-cardiac angina-like” chest pain Pyrosis or heartburn, pharyngeal burning Belching, postprandial fullness Irritable oesophagus General irritability (infants)
Unusual presentations GER related to chronic respiratory disease (bronchitis, asthma, laryngitis, pharyngitis, etc.) Sandifer Sutcliffe syndrome Rumination Apnea, apparent life threatening event and sudden infant death syndrome	
Associated to congenital and/or central nervous system abnormalities Intracranial tumors, cerebral palsy, psychomotory retardation	

A number of these symptoms may also be caused by other mechanisms.

Table 2(PDF) Effect of special formula and milk-thickening products on GOR, gastric emptying (GE) and clinical parameters in infants with GOR disease (=: unchanged, ＜: worse, ＞: better)

[a]means of age in groups, [b]O: open, SB: single blind, XO: crossover, PA: parallel, [c]thickened meal (FT) vs unthickened meal (noFT) or vs baseline (B) or comparison of special formula, cn.d.: no data, N.S.: not significant.

Table 3(PDF) Effects of cisapride (CIS) on GOR disease in infants

[a]Age: mean(s) of age in group(s); [b]O: open; DB: double bl ind; PA: parrallel; XO: cross-over with wash-out period; [c]CIS: cisapride ; PLA: placebo; MCL: metoclopramide; DO: domperidone; CIM: cimetidine; GAV: Gavi scon; AA: antacid; FT: feed

d

thickener; B: baseline; CO: controls; bm: before mea ls/each feeding; afm: after meals; DM: dietary measures; SD: standard diet; dex: dextrose; glu: glucose; CF: customary formula; SF: solid food started if not yet done so; PN: parenteral nutrition; *prior therapeutic measures co ntinued (positional and/or dietary); e,f-Outcome: GOR, reflux parameter s on pH monitoring; GE, gastric emptying; ＞, better than; ＜, worse than; =, un changed, referring to the main/all parameters evaluated in paper; exceptions for single parameters are mentioned separately. Symptoms: if not specified, clinica l assessment including regurgitation and/or vomiting. PLES: pressure lower esoph ageal sphincter. n.d.: no data.

Table 4 Contraindications and risk factors for use of cisapride

Contraindications to cisapride administ ration in pediatric patients
-Combination with medication also known to prolong the QT interval or potent CYP3A4 inhibitors, such as astemizole, fluconazole, itraconazole, ketoconazole, miconazole, eythromycin, clarithromycin, troleandomycin, nefazodone, indinavir, ritonavir, josamycin, diphemanil, terfaridine. -Use of the above medications by a breast-feeding mother, as secretion i n mother's milk of most of these drugs is unknown. -Known hypersensitivity to cisapride. -Known congenital long QT syndrome or known idiopathic QT prolongation.
Precautions for cisapride administration in pediatric patients
-Prematurity (a starting dose of 0.1mg/kg,4 times daily may be used, although 0.2mg/kg is also for prematures the normal dose) -Hepatic or renal failure (particularly when on chronic dialysis). In these cases, it is recommended to start with 50% of the recommended dose. -Uncorrected electrolyte disturbances (hypokalemia, hypomagnesemia, hypocal cemia), as may occur in prematures, in severe diarrhea, in treatment with potass ium-wasting diuretics such as furosemide or acetazolamide. -History of significant cardiac disease including serious ventricular arr hythmia, second or third degree antrioventricular block, congestive heart failur e or ischaemic heart disease, QT prolongation associated with diabetes mellitus. -History of sudden infant death in a sibling,and/or history of a“serious ”apparent life threatening event in the infant or a sibling.-Intracranial abnormalities, such as encephalitis or haemorrhage, grape fr uit juice.

In the non-breast-fed infant, a change to a (thickened) hydrolysate or amino- acid formula should be considered, if regurgitation is resistant to a thickened formula with normal proteins and to prokinetics, since protein allergy may prese nt as therapy-resistant GER-disease.

Non-drug treatment (positional therapy, dietary advice) can help convince the p arents of the physiologic nature of the regurgitations [3]. The influence of position on the incidence and duration of GER episodes has been demonstrated in adults, children and infants both in asymptomatic healthy controls and sympt omatic individuals. The 30° prone reversed Trendelenburg position is nowadays generally recommended and accepted as an essential element of treatment [3,6,7]. However, positional treatment is in practice very difficult to apply correctly in infants and rather unfriendly to the babies, since they have to be tied up in their beds or cot to prevent them from sliding down under the blankets, since an angle of 30° has to be achieved and maintained. The ample evidence that the prone sleeping position is a risk factor in sudden infant death,

independent of overheating, smoking or way of feeding [6]. Positional treatment remains, in view of its efficacy, a valid "adjuvant" treatment in patients not responding to other therapeutic approaches or beyond the age of sudden infant death [6].

Group 2. Overt GER-disease

Patients in this group did not either respond to the previous approach (parental reassurance, dietary treatment and prokinetics) or present with symptoms suggesting esophagitis (hematemesis, retrosternal, epigastric pain, etc.) (Table 1). Therefore, an underlying anatomic malformation should be excluded, and endoscopy is the investigation of choice [3,19]. Upper gastrointestinal endoscopy in infants and children should only be performed by experienced and qualified physicians [19]. If the question being asked is restricted to underlying anatomic malformations, upper gastrointestinal series can be considered [19]. If symptoms and/or the esophagitis do not improve despite adequate medic al treatment and controlled compliance, upper gastrointestinal series should be performed to exclude anatomical problems such as gastric volvulus, intestinal ma lrotation, annular pancreas, etc.

Antacids are reported to be effective in the treatment of GER [6], although experience is limited in infants. Their capacity to buffer gastric acid is strongly influenced by the time of administration [20], and requires multiple doses. Gaviscon (a combination of an antacid and sodium salt of alginic acid) is as effective as antacids and appears to be relatively safe, since only a limited number of side effects have been reported. Occasional formation of large bezoar-like masses of agglutinated intragastric material has been reported with the use of Gaviscon, and it can increase the sodium content of the feeds to an undesirable degree especially in preterm infants (1g Gaviscon-powder contains 46mg sodium, and the suspension contains twice this amount of sodium) [6].

H^2-receptor antagonists, of which ranitidine is by far the mostly used, are e ffective in healing reflux esophagitis in infants and children [6]. Many new drugs have been developed (misoprostil, sucralfate, omeprazole, etc.). Of these, the proton pump inhibitors (PPIs) have been studied best, although experien ce in infants and children is limited [21,57]. PPIs are effective in supp ressing the acidity in patients with gastric stress ulcer(s) and also in neurolo gically impaired children. Even in patients with circular esophageal ulcerations , recent experience suggests that PPIs should be given a chance prior to surgery [21]. Omeprazole is known to be effective in patients with severe esophagitis refractory to H^2 blockers [21]. Sucralfate was shown to be as ef fective as cimetidine for esophagitis in children [22].

Immediate or early surgery is rarely indicated in life threatening conditions where medical management will be of no benefit. Surgery can be life-saving in severely affected patients (notably the neurologically impaired children with recur rent and life-threatening aspiration, etc.). Prior to surgery, a full diagnosti c work-up including upper gastrointestinal series, endoscopy, pH monitoring, eventually completed with manometry and gastric emptying studies is recommended.

Group 3. Patients with unusual presentations of GER

The most obvious difference between this patient group and groups 1 and 2, is that this patient group does not present with emesis and regurgitation (Table 1). Since these patients do not vomit, GER-disease is "occult". Before considering GER as a cause of the symptoms, classic causes of the manifestations need to b e excluded, such as allergy in a wheezing patient, tuberculosis in a patient wit h chronic cough, etc.

If GER-disease is suspected, pH monitoring of long duration (18-24 hours) is the investigation of choice. In this group of patients, pH monitoring may need t o be performed in simultaneous combination with other investigations in order to relate pH changes to events (e.g. polysomnography in the infants presenting with an apparent life threatening event). In patients suspected of pulmonary aspiration, a scintigraphy might prove the association (although a negative scintigraphy does not exclude reflux related aspiration, and the therapeutic approach will be identical).

If pH monitoring is abnormal or if events are clearly related to pH changes, prokinetics, eventually in combination with H^2 receptor antagonists or PPIs, are indicated [19,21] .In this group, repeat pH monitoring under treatment conditions in combination with a clinical follow-up is mandatory. Depending on the unusual presentation, treatment can be stopped after 6 to 12 months, since a possible mechanism for GER in association with unusual manifestations may be self-perpetuating GER [23] . Once reflux occurs, acid gastric contents containing pepsin and sometimes bile comes into contact with the esophageal mucosa, which increases the esophageal permeability to acid and makes the esophageal mucosa much more susceptible to inflammatory changes. Esophageal inflammation, even res tricted to the lower esophagus, impairs LES pressure and function, and favors GE R [23] .

Severely neurologically impaired children

The vast majority of neurologically impaired children suffer from severe GER- disease. Most of these children are under specialized follow-up, and only brief recommendations will be given here. The pathophysiological mechanism of GER-di sease in these children is particularly multifactorial: the neurological disease itself (which might cause delayed esophageal clearance and delayed gastric empt ying), the fact that most of these children are bedridden (gravity improves esop hageal clearance), many are constipated (which increases abdominal pressure and favors GER), etc.

CONCLUSIONS

The diagnostic approach of GER(-disease) in infants and children principally de pends on its presenting features. Infants with typical symptoms of uncomplicated GER (the majority of regurgitating babies) should be treated without prior investigations. Endoscopy, in specialized centers, is recommended if esophagitis is suspected. Long-term esophageal pH monitoring is the investigation of choice and occupies a central position in the diagnostic approach of the patient suspected of unusual or atypical presentations of GER- disease (“occult” GER-disease). Non-drug treatment (the importance of parental reassurance cannot be stressed enough) and dietary treatment are an effective and safe approach in infant regur gitation, but does not treat GER-disease. If the symptoms are refractory to thi s approach, or in reflux-disease, cisapride is the drug of choice. PPIs or H^2-receptor antagonists, in combination with prokinetics, are recommended in (ulc erative) esophagitis. There is no excuse to persist with an ineffective manageme nt of a disease which might result in stunting, chronic illness, persistent pain , esophageal scarring or even death. Management of GER(-disease) in infants and children should therefore be well overthought, avoiding overinvestigations and o vertreatment of a self-limiting condition, but also avoiding underestimation of potential severe disease, accompanied by serious morbidity.

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Reassessment of barium radiographic examination in diagnosing gastrointestinal diseases

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Subject headings gastrointestinal radiography; gastrointestinal diseases/radiography; barium radiographic examination

Gastrointestinal radiography (GIR) has been a major and first-choice method for diagnosing gastrointestinal diseases with barium as contrast media since its emergence in 1910, even in diagnosing the mass lesions of the org ans outside gastrointestinal tract (e.g., liver, pancreas, etc) indirectly. The fiber endoscopy invented in the late 1960s can directly observe the changes on mucosal surface intraluminally and obtain biopsies as well, thus greatly improving the detection and sensitivity of small, shallow or tiny lesions originated from mucosa. This discovery is a big challenge to GIR, which has not only profoundly modified the dominant role of GIR in diagnosing gastrointestinal diseases, but also aroused different viewpoints and evaluations even more radically[1]. According to the data collected from 69 radiologic units during the 12 years by the American Society of Gastrointestinal Radiography, the number of upper GIR decreased by 24%, colon examinations decreased by 29%, averaging 25%[2]. The situation is similar domestically. Some GI radiologists even gave up their experienced studies in GIR. Young radiologists are just eager to study the new inventions such as CT and MR, and neglect GI. All those interfered with and even lowered the quality and quantity of GIR. This is one aspect of the problem.

On the other hand, the invention of endoscopy has positive influence on GIR. It has made many GI radiologists realize that lots of diseases can not be detected with the single contrast GIR, which can not meet the demand of therapy and must be improved. In the 1960s, Japanese scholars developed and created the brand new double-contrast GI technique on the basis of the traditional single-contrast GIR[3], meanwhile, through studies on barium contrast media, assistant agents (mucus detergent, gas agent)[4,5], radiologic technique, the methods and the principles of imaging[6,7], great achievements in practice and theory have been made (Figure 1)[8]. The double-contrast GIR became the universal method in the 1980s, the sensitivity of double-contrast GIR in diagn osing early cancer[9,10], superficial erosion and linear ucler become higher and higher[11]. It made it possible to observe the locations such as cardia which can be hardly shown by single-contrast GIR much easier for diagn osis[12,13], thus re-establishing the status of GIR with high prestige. But it is a regret that this achievement could not be popularized nationwide.

The multiphase gastrointestinal radiography (MPGIR) can not only diagnose shallow mucosal lesions as endoscopy, but also has its own special effects that other approaches can not possess in diagnosing gastrointestinal diseases[14].

AN IDEAL FIRST-CHOICE DIAGNOSTIC MODALITY FOR GI TRACT

It has been well known that the different diseases of gastrointestinal tract usu ally present the similar symptoms, which are atypical. Clinical doctors can hardly ascertain the exact location of the lesions when making the diagnoses, and the physical diagnostic approaches are usually necessary. The symptoms caused by these lesions include the following aspects: most of them originated from the mucosa of the gastrointestinal tract, such as inflammation, erosion, ulcer and cancer, etc. (Figure 2); some originated from the submucosa, such as nonepithelial (e.g. smooth muscle, fat and nerve) benign or malignant tumors[15]; some are functional disorders[16], such as reflux disorder, achalasia etc.; some are of organic deformation, such as various kinds of organ volvulus, diverticulum and hernia; and some originated from outside gastrointestinal tract (e.g., pancreas, gall bladder, ovarium and uterus). It is doubtless that the first choice for physical diagnosis is to judge several aspects of these lesions mentioned above simultaneously. MPGIR which possesses the advantage of both single-contr ast and double-contrast GIR, can not only examine organ morphologic changes caused by the diseases

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themselves or adjacent organs, but also identify organic functional changes. It can satisfactorily perform various examinations for the whole gastrointestinal tract, including multiphasic hypotonic upper gastrointestinal radiography, small bowel enema SBE, GI series, and double contrast enema DCE. That can meet the demand of clinical doctors as the first choice examination.

AN OVERALL DIAGNOSTIC MODALITY FOR GI TRACT

For the needs of planning the therapy and predicting prognosis correctly, four targets must be considered: site, sickness (qualitative determination), structure (quantitative determination), staging. That is the so called four "S" diagnosis. The basis of four "S" diagnosis is pathomorphologic and pathophysiologic changes[17]. All of the physical examinational approaches (endoscopy, radiography, ultrasonography and endoscopic ultrasonography, MR, etc.) are all expe cted to reflect the pathologic changes objectively, and all kinds of examinational methods must be based on these criteria.

Refined MPGIR, which can obtain a complete view of the motion, the adoption, the ejection of organs and the pathophysiologic situation of the diseases, especially the location, shape, pathologic changes (different size, degree and extent) caused by mucosal or submucosal lesions, is superior to other techniques[18].

To detect the lesion-site

With full use of filling phase and double-contrast phase, MPGIR can easily obtain a complete view of the organ. Based on this, it can detect the abnormality of organ location, size and shape, making it easy to diagnose organ volvulus, diverticulum, hernia and so on[14]. According to the different organic changes caused by the lesions and the correlation between lesions and organs, MPGIR can clearly establish the origination (such as from the organ itself, the intramural or the extramural) of lesions for most of the patients. It can also show the pattern of lesions, whether protruding or depressing or both.

To determine the nature of the lesion-sickness

The nature of the lesion of gastrointestinal tract can be determined by its specific signs which appears in MPGIR under different contrast conditions (filling, mucosal, double-contrast, compressed phase) (Figure 3). Many these effective signs have been found by the radiologists, such as "bull eyes sign" indicating submucosal leiomyoma, "target sign" meaning erosion, "dimness, coarse, rigid sign" hinting malignant tumor, "step-like mucosal pattern" suggesting early gastric cancer, etc[8]. The better technical quality the more truthful im age and the more specific appearance the more accurate diagnosis[15,19,20].

To identify the degree of the lesion-structure

Inflated by gas, the lesion outlined by barium is very clear and complete in MPGIR. It is very valuable for judging the size, extent and structure of lesions[14]. For example, in Borrmann's type I cancer, it can clearly provide the overview of the size and the shape of the tumor protruded into the lumen, and can also obviously survey the gastric wall involved by the tumor, which is bene ficial for surgery (Figure 4). Multiple primary carcinoma (MPC) of gastrointesti nal tract is not rare, it can occur at either the same organ, such as multiple esophageal carcinomas, multiple gastric carcinomas or different organs, such as multiple esophagogastric carcinomas and multiple esophagogastrorectal carcinomas [21]. Preoperative discovery and diagnosis of all these lesions are espe cially important. It is less difficult to make diagnosis for MPGIR if more atten tion is paid to it. Nevertheless, other physical diagnostic approaches can hardly detect all the lesions, which might cause fatal results. For diagnosing gastro intestinal benign or functional disorders, the concept of "structure" still exists, such as evaluating the number and the size of ulcers, the type and the volume of reflux by using MPGIR.

To ascertain the stage of the lesion-staging

MPGIR can sometimes ascertain the stage of the lesion in diagnosis. A niche suggests "active period" of the ulcer, a linear barium shadow means "healing period", and a scar formation indicates "healed period" of the ulcer (Figure 5)[11]. To stage of carcinoma, the present signs can be used for initial judgement, for example, the special changes of mucosal folds imply early cancer, the "stiff gastric wall" sign suggests advanced cancer (Figure 6)[8]. However, the preoperational staging of cancers should be combined with CT examination[17,22].

THE INNOVATION OF GASTROINTESTINAL (GI) RADIOGRAPHY TECHNIQUE

The traditional GI radiography has experienced the following periods: local-con trolled fluoroscopic radiography, remote-controlled image intensifier, TV display, spot film[1], etc. In recent years, with the development of high-speed and high-efficiency computer, radiologic diagnosis has gradually become a new filmless technique using digital and postprocessing imaging systems. Digital GI imaging set on the basis of satisfactory double contrast GI technique possesses many advantages as follows which routine spot-film and fluororadiography do not have[23,24].

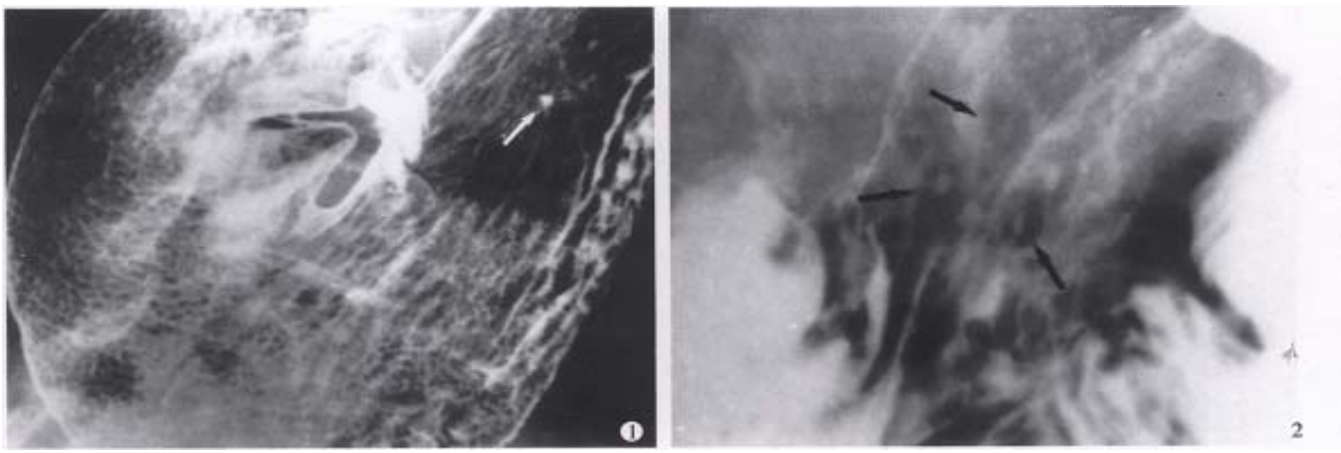

Figure1 Areae gastricae. Well-circumscribed polygonal radiolucencies surrounded by barium-filled shallo w grooves are clearly seen on the mucosal surface. A small benign ulcer is also displayed (arrowhead).
Figure2 Erosive gastritis. Multiple varioliform erosions (arrows) in the posterior wall of gastric body are seen as tiny barium collections with surrounding halos of edematous mucosa-"target" sign.

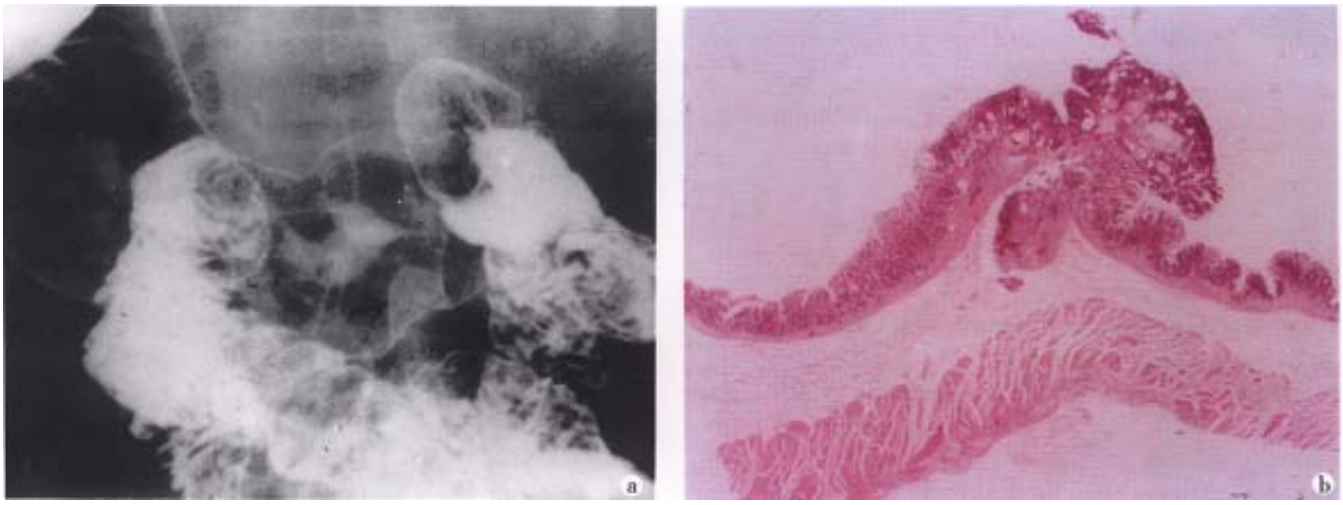

Figure3 Small early gastric cancer (type I). A sessile protruded lesion (5mm in size) with i ts base puckered into the lumen is seen on the greater curvature of antrum.B. Micrograph shows the lesion originates from mucosa and infilt rates to the submucosal layer.

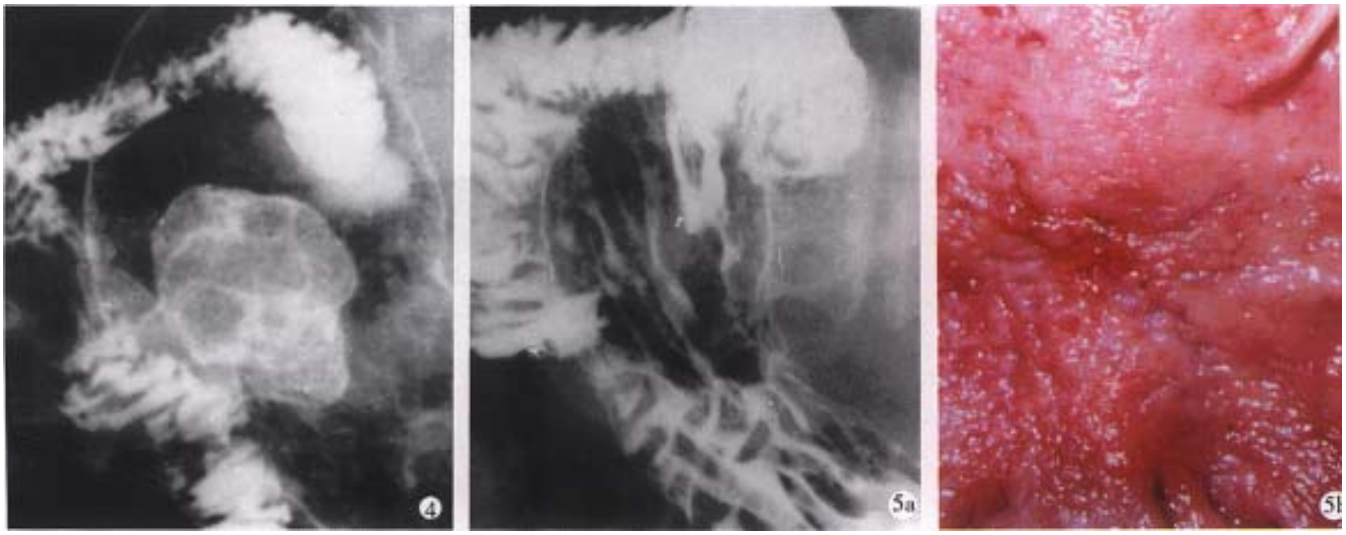

Figure4 Gastric carcinoma (Borrmann's type I).
A bulky cauliflower-like mass etched in white by a thin layer of barium is seen on the greater curvature of the stomach.
Figure5 Linear ulcer. A long linear ulcer paralleled to the lesser curvature (ar rowhead) shown on the gastric posterior wall, representing the healing and heale d stage of ulcer. B. Macrograph shows that the fine curve linear ulcer is compo sed of re-epithelialized (healed) and granulating part (healing).

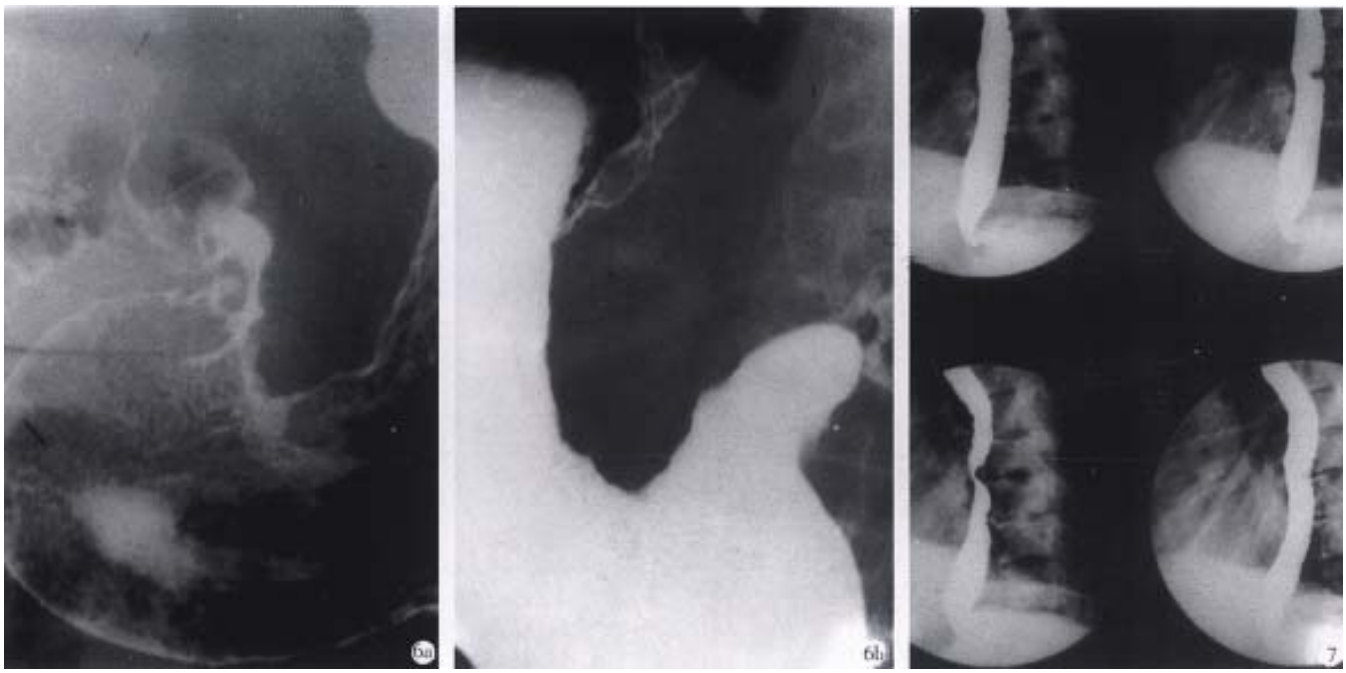

Figure6 Advanced gastric carcinoma. A. Double-contrast phase. B. Filling phase reveal marked "rigid" and "coarse" contour of the lesser curvature and lumen narrowing caused by infiltrative carcinoma.
Figure7 Digital esophageal barium radiography. The defect on posterior aspect of middle esophagus caused by the proliferation of thoracic vertebrae is easily displayed by rapid sequence images (0.5 picture s / sec) and results in dysphagy clinically.

Rapid sequence (0.5-15 pictures/sec) images

Rapid sequence images is very helpful for surveying the moving gastrointestinal tract, especially in the oral pharynx and the upper esophagus. As sufficient esophageal dilatation and mucosa coating can only keep on comparatively a short time, with the pulsation of heart, the high-quality D.C. films are not easy to acquire. Digital sequence exposure (0.5-2 pictures/sec) which can overcome these deficiencies, is obviously better than traditional imaging technique, for obtainning multiphasic images of esophagus (Figure 7)[25].

Quality control for the study

Every continuous picture can be accommodated on the monitor as a "frozen image", until the next fluoroscopy begins. It can give the operator a clue whether the lesion is displayed satisfactorily[24]. If not, the doctor can adopt their apt procedure: changing projective position, mucosa recoating, and some more digital images in order to avoid the lesions missed due to slipshod examinational technique. Therefore, it can guarantee the quality of MPGIR, and increase the detectability of lesions.

Postproccessing

Digital imaging is not the same as the routing radiography, which can change the parameters in workstation, regulate the brightness and contrast of the picture, magnify the interesting area, adjust the edge enhancement, and annotate and print findings, and enter the picture archiving and communication system (PACS). All of these are making MPGIR enter into a brand new information era.

In summary a high-quality MPGIR can not only diagnose gastrointestinal diseases or disorders, but also make more complete judgement at the aspects of "site", "sickness", "structure" and "stage" provided by MPGIR[14,18,22]. It is charaterized by its own speciality and advantages: the convenient operatio n, and less sufferings caused to patients. So, it is still an important and the first choice examination. Both MPGIR and endoscopy must be adopted in a compleme ntary manner to make up each other's deficiencies, but not to be replaced one another. Of course, it is essential to maintain and improve the quality of MPGIR constantly. It is gratifying that the use of MPGIR examinations is increasing by 9.8% after 1996 in spite of its decline in the earlier 1990s according to the data from three big comprehensive hospitals in Shanghai. The current gastroi ntestinal radiology is one of the valuable medical treasures accumulated by many generations of scientists[8]. We should practise it continuously and explore, develop and improve it constantly, and do our best to contribute to gastr ointestinal radiology.

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Fibrodynamics-elucidation of the mechanisms and sites of liver fibrogenesis

Catherine H. Wu, Ph.D.

See article on page 397

Subject headings liver cirrhosis/pathology; liver cirrhosis/etiology; liver cirrhosis/physiopathology; fibrodynamics; hepatic stellate cells

ORIGINAL ARTICLE

Dynamic changes of type I, III and IV collagen synthesis and distribution of col lagen-producing cells in carbon tetrachloride- induced rat liver fibrosis.

MAJOR POINTS OF THE COMMENTED ARTICLE

In their article that appears in this issue, Du and colleagues used a combination of immunohistochemistry and *in situ* hybridization to demonstrate increased levels of collagen types I, III and IV in CCl_4/choline deficient rat model of hepatic fibrosis. Over the course of 20 weeks of treatment mRNA levels for all three types of collagen were increased, but there was a preferential increase of type III collagen mRNA over the other two types. This is consistent with the results of previous investigators[1,2] where increases in protein levels of collagen types I, III and IV were found in CCl_4 induced liver fibrosis. The authors clearly demonstrated that collagen type I, III and IV mRNAs were localized on sinusoidal cells using *in situ* hybridization. This result is also consistent with previous work of Maher and co-workers[3,4] who used the same technique to demonstrate the localization of both interstitial and basement collagen mRNAs in hepatic stellate cells in normal rat and human livers. The aut hors have utilized state-of-the-art technology to examine an important questi on in liver fibrosis-the cells responsible for overproduction of liver biomatrix components. Their results are consistent with the results of other investigato rs in the field. However, caution should be taken not to overinterpret results. Additional controls in which the *in situ* localization of collagen mRNAs in untreated normal liver as compared to that seen in fibrotic liver could have given a clearer picture of changes in the fibrotic liver. Nevertheless, the results add strong confirmation to the temporal course of fibrogenesis, and localization of the products in an important model.

COMMENTARY

Liver fibrosis is the common result of chronic hepatic injury of diverse origins such as chronic viral infections (HBV, HCV), metabolic/storage diseases (hemochromatosis), helminthic infections (schistosomiasis), chronic toxin exposure (alcohol and environmental poisons) and biliary obstruction (biliary cirrhosis). In end stage liver fibrosis or cirrhosis, the liver biomatrix may contain up to six to ten times more collagen and proteoglycans than in the normal state[5,6]. Because the connective tissue support of the liver parenchyma is particularly critical to its function, research that emphasizes the nature of liver biomatrix, the molecular regulation of the tumover of components of the biomatrix, and identification of liver cells responsible for the synthesis of biomatrix proteins are especially crucial for the ultimate design of effective therapies for liver fibrosis.

In the late 50's, Hans Popper, the eminent hepatologist, observed a correlation between the histomorphology and biochemistry of liver collagens in chronic liver diseases[7]. In the four decades following Dr. Popper's original obser vation, a great deal of research on liver biomatrix has resulted in our current knowledge of the pathogenesis of liver fibrosis. Progress has been made in three areas of liver fibrosis: characterization and quantitation of matrix component s in normal and fibrotic liver; identification of hepatic cells responsible for the increased synthesis of matrix proteins; and the role of cellular mediators of fibrogenesis.

Quantitation of matrix proteins in normal and fibrotic livers

The use of animal models of liver fibrosis such as the administration of liver toxins CCl_4[8], dimethylnitrosamine[9], alcohol[10], helminthic

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infections[11,12] have greatly helped in the characterization of the temporal expression of various components of the biomatrix during fibrogenesis. There is increase in the amounts of collagens types I, III, IV, V and VI[1]. In early fibrosis, the amounts of types III and IV collagens increase relative to other collagens. In late fibrosis, type I collagen predominates[13]. Other components of liver biomatrix such as laminin, fibronectin and proteoglycans are also increased in fibrosis[14,15]. Although most investigations have shown changes at the protein and mRNA levels of various biomatrix components, the significance of the changes in the fibrogenic process remain hotly debated[16]. The biomatrix in the normal liver changes from bein g rich in basement membrane collagens to interstitial collagens during fibrogenesis.

Hepatic stellate cells is the major effector cell type in hepatic fibrosis

The search for effector cells in the liver responsible for collagen synthesis became feasible with the development of molecular probes[17] and antibodies[14,18] to components of liver biomatrix. Stellate cells are responsible for the increased synthesis in liver biomatrix proteins such as basement membrane collagens, interstitial collagens, fibronectin, laminin and proteoglycans[3,4]. Activation of hepatic stellate cells is the earliest response to liver injury. Upon activation, stellate cells lose stored lipids and retinoids[19] and rapidly undergo morphological changes to myofibroblast-like phenot ype[20]. Other phenotypic changes of activated stellate cells include st imulation of α-actin gene expression[21,22] and increased synthesis of hepatic biomatrix components[3]. Activation also results in loss of an important feedback regulation of collagen synthesis by its terminal propeptides. In the normal liver, stellate cells are capable of controlling the amount of co llagen needed for normal biomatrix formation by a feedback inhibition of collage n synthesis by its terminal propeptides[23,24]. Following activation, stellate cells lose their normal feedback regulation of collagen synthesis leading to increased accumulation of collagen[25]. In particular, there is an increased synthesis of types I and III collagen resulting in a biomatrix rich in interstitial collagens. There is increasing evidence that accumulation of fibers in the sinusoids is not only due to increased synthesis of collagens, but also is a result of decreased synthesis of tissue collagenases and increased synthesis of inhibitors of collagenase (TIMP-1: tissue inhibitors of metalloproteinase)[26]. Thus fibrogenesis is a net result of increased synthesis and decreased degradation of interstitial collagens of activated stellate cells.

Cellular mediators of hepatic fibrosis

Understanding the underlying molecular mechanisms responsible for hepatic fibrosis became feasible with the availability of molecular probes to cytokines. It is now accepted that the initial liver injury results in a host of cytokine responses from liver cells. Specifically, TGFβ[27], TNFα[28], PDGF[29] and Kupffer cell soluble factors[30] have been implicated in stellate cell activation and proliferation. TGFβ mRNA and protein levels are increased in activated stellate cells[27]. Over-expression of TGFβ gene in cultured fibroblasts[27] and in stellate cells[31] results in increased synthesis of collagens. Inhibitors of TGFβ decrease collagen synthesis *in vivo*[32,33] while transgenic mice over expressing TGFβ have kidney and liver fibrosis[34]. Both PDGF[29] and TNFα[35] are stellate cell mitogens. PDGF-induced stellate cell proliferation and matrix protein synthesis is mediated by factors secreted by Kupffer cells[30]. TNFα acts via transcription regulation of tissue collagenase and TIMP-1 genes in activated stellate cells[28].

Current research

The elucidation of the molecular mechanisms of cytokine regulation of liver biomatrix protein synthesis continue to be a focus of current research efforts. There is increasing evidence that cytokines may act via interactions with DNA binding proteins to affect matrix proteins synthesis. Both TGFβ and TNFá interact with known transcription factors such as C/EBP[36] and NFkappaB[37,38]. Transcription factors are DNA binding proteins which act as regulators of gene transcriptions[39,40]. There is continued interest in the search for regulatory elements within genes of matrix proteins[41]. Research on interactions of DNA binding proteins to regulatory elements on matrix protein genes are underway and may provide a link between cytokines and regulation of liver biomatrix.

Future directions

Effective therapy for chronic hepatic fibrosis can be designed only with complete understanding of the molecular mechanisms that regulate matrix protein gene expression. Future research may be centered on the application of gene therapy to control hepatic fibrosis[42]. Over-expression of tissue collagenase gene, inhibition of TGFβ gene expression are potential approach in controlling and regulating hepatic fibrosis[43].

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Original Articles

Increased prevalence of intestinal inflammation in patients with liver cirrhosis

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Subject headings liver cirrhosis; intestinal; inflammation; s ecretory IgA; fecal proteins

Abstract

AIM To investigate the pathophysiology of the digestive tract in patients with liver cirrhosis.

METHODS In 42 cirrhotic patients and 20 control subjects, the following fecal proteins were measured by enzyme-linked immunosorbent assay: albumin (Alb), transferrin (Tf), and α_1-antitrypsin (α_1-AT) as a marker for intestinal protein loss, hemoglobin (Hb) for bleeding, PMN-elastase for intestinal inflammation, and secretory IgA for intestinal immunity.

RESULTS The fecal concentrations of Hb, Alb, Tf, α_1-AT, an d PMN-elastase were increased in 13 (31%), 8(19%), 10(24%), 6(14%), and 11 (26%) cases among 42 patients, respectively. Fecal concentration of secretory IgA was decreased in 7 (17%) of 42 patients. However, these fecal concentrations were not related to the severity or etiology of liver cirrhosis. The serum Alb level was significantly decreased in patients with intestinal protein loss compared to that in patients without intestinal protein loss.

CONCLUSION These findings suggest that: ① besides the well- known pathological conditions, such as bleeding and protein loss, intestinal inflammation and decreased intestinal immunity are found in cirrhotic patients; ② intestinal protein loss contributes to hypoalbuminemia in cirrhotic patients, and ③ intestinal inflammation should not be over looked in cirrhotic patients, si nce it may contribute to or cause intestinal protein loss and other various path ological conditions.

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INTRODUCTION

Gastrointestinal symptoms are common in patients with liver cirrhosis and portal hypertension. Their pathophysiology remains, for the most part, obscure. It is well known that esophageal varices and portal hypertensive gastropathy[1,2] are common causes of upper gastrointestinal (GI) bleeding in patients with liver cirrhosis. Recently, portal hypertensive colopathy, such as colonic vascular ectasia and rectal varices, were recognized as causes of lower gastrointestinal bleeding in cirrhotic patients[3,4]. Other than bleeding, various pathological conditions such as malabsorption[5,6] and intestinal protein loss[7,8] have been reported in cirrhotic patients. We developed fecal tests that are useful for evaluating the various pathophysiologies of the digestive tract[9-12]. Fecal hemoglobin (Hb) is a useful marker of bleeding. Fecal albumin, transferrin (Tf), and α_1-antitrypsin (α_1-AT) are useful markers of intestinal protein loss. Fecal secretory IgA (sIgA) level reflects the condition of local immunity in the intestine and an increase in fecal neutrophil granule-derived proteins, such as PMN-elastase, indicates the presence of intestinal inflammation. In the present study, we examined the fecal protein profile to investigate the pathophysiology of the digestive tract in cirrhotic patients.

MATERIALS AND METHODS

Subjects

Forty-two patients with liver cirrhosis aged 59.0± 8.7 years (mean ± SD) were evaluated. The diagnosis of liver cirrhosis in each case was confirmed by a comb ination of clinical, biochemical, radiological, and pathological methods. The clinical severity of the liver disease in each case was determined using the Pugh modification of Child's original classification[13]. These cases consisted of thirty-one males and eleven females. The number of patients with Child A, Child B, and Child C were 27, 6, and 9, respectively. The etiology of the liver cirrhosis was as follows: 33 patients with hepatitis C, 3 with hepatitis B, and 6 with alcoholism. All patients had

no obvious gastrointestinal bleeding. The control group consisted of 20 subjects aged 41.0±18.1 years with no demonstrated abnormality in the upper or lower digestive tract. Informed consent was obtained from each subject in accordance with the Helsinki Declaration.

Stool collection and measurement of fecal proteins

Patients were instructed to defecate into a polystyrene container (diameter 15 cm, depth 12 cm). The stool samples, collected at 4 ℃ over a period of 48 h - 72 h, were homogenized with a small amount of water, then sto red at -80 ℃ until the time of measurement. The concentrations of fecal Hb, Alb, Tf, α_1-AT, PMN-elastase, and sIgA were measured by enzyme -linked immunosorbent assay (ELISA) as described previously[9-11]. Brie fly, anti-human Hb antibody (Institute of Immunology, Japan), anti-human albumin antibody (Dakopatts, Glostrup, Denmark), anti-human Tf anti body (Dakopatts), anti-human α_1-AT antibody (Dakopatts), anti-human PMN-elastase antibody (Serotec, Oxford, England), or anti-human secretory component antibody (Dakopatts) was coated in the wells of a 96-well microplate. The diluted fecal samples (100 to 10000 fold) were added to each well. After reaction at 37 ℃ for 1 h, the wells were washed with water . The samples were then reacted with the respective alkaline phosphatase- labeled antibody except alkaline phosphatase labeled anti-human IgA antibody (Dakopa tts) for ELISA of sIgA. The enzyme reaction was then carried out, and color deve lopment was measured with a microplate colorimeter at 510/630 nm.

Statistical analysis

Values were expressed as medians (25%, 75%). The Mann-Whitney U tests and/or χ^2 tests were used to compare groups. All *P* values were two-tailed; *P* values less than 0.05 were considered statistically significant.

RESULTS

Fecal protein concentrations in control subjects

Results are shown in Figure 1. Daily stool weight (g/day) was 140 (100, 230) in control subjects. The concentrations of fecal Hb, Alb, Tf, α_1-AT, sIgA, and PMN-elastase were 1.5 (0.1, 4.0), 0.1 (0.1, 1.8), 0.1 (0.1, 0.4), 327.4 (201.1, 421.3), 214.9 (56.6, 244.1), and 0.6 (0.3, 1.2), respectively. The 95th percentile of the control subjects was used as the cu t-off value (10.6, 5.3, 1.0, 771, and 2.2 μg/g for Hb, Alb, Tf, α_1-AT, and PMN elastase, respectively). sIgA was important when not only increasing, but also decreasing. Therefore, the lower limit of the normal range was defined as the 5th percentile (26.8 μg/g) of the control subjects for sIgA.

Fecal protein concentrations in cirrhotic patients

Daily stool weight (g/day) was 140 (100, 180). In terms of daily stool weight, there was no significant difference between the cirrhotic patients and control subjects. As shown in Figure 1, the fecal concentrations of Hb, Alb, Tf, α_1-AT, and PMN-elastase were increased in 13 (31%), 8(19%), 10 (24%), 6(14%), and 11 (26%) among 42 patients, respectively. Conversely, the fecal con centration of sIgA was decreased in 7 (17%) of 42 patients. There were significa nt differences in fecal Hb, Alb, sIgA, and PMN-elastase concentrations between the cirrhotic patients and the controls.

Relationship between fecal protein concentrations and the severity of liver cirrhosis

As shown in Figure 2, there was no relationship between the concentrations of these fecal proteins and the severity of the disease.

Relationship between fecal protein concentrations and the presence of esophageal varices

As shown in Figure 3, fecal Hb concentration demonstrated a significant difference between the cirrhotic patients and the controls.

Relationship between fecal protein concentrations and the etiology of liver cirrhosis

There was no association between fecal protein concentrations and the etiology of the liver disease (data not shown).

Intestinal protein loss and nutritional status in cirrhotic patients

Results are shown in Figure 4. The patient was defined as having intesti nal protein loss, when a patient had increased concentrations of at least one of Alb, Tf, and α_1-AT. The serum Alb level was significantly decre ased in patients with intestinal protein loss compared to that in patients without intestinal protein loss. The findings suggest that intestinal protein loss contribute to the development of hypoalbuminemia in cirrhotic patients.

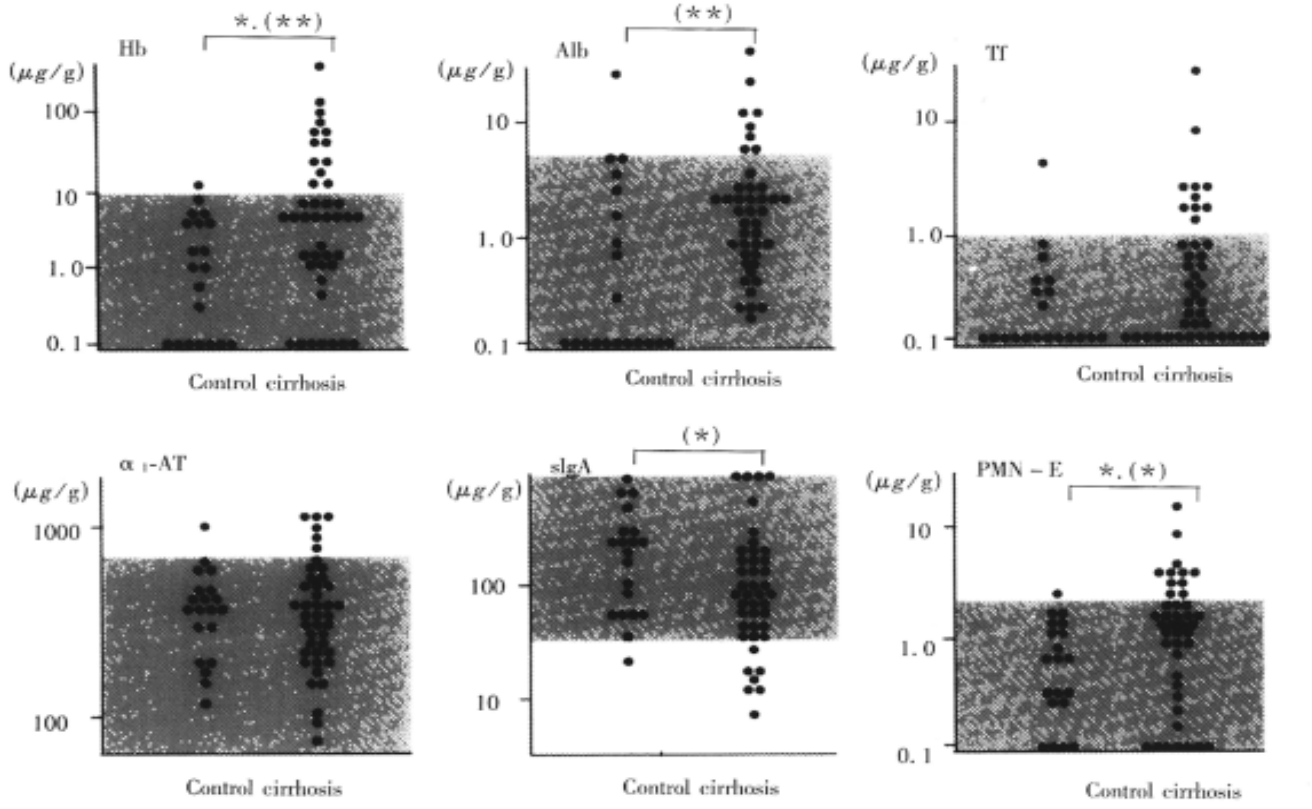

Figure1 Fecal protein concentrations in cirrhotic patients and control subjects. The dotted area shows the 95 percentile of the c ontrol subjects. [a]: $P<0.05$ by c^2 test; [b]: $P<0.05$; [c]:$P<0.01$ by Mann-Whitney U test.

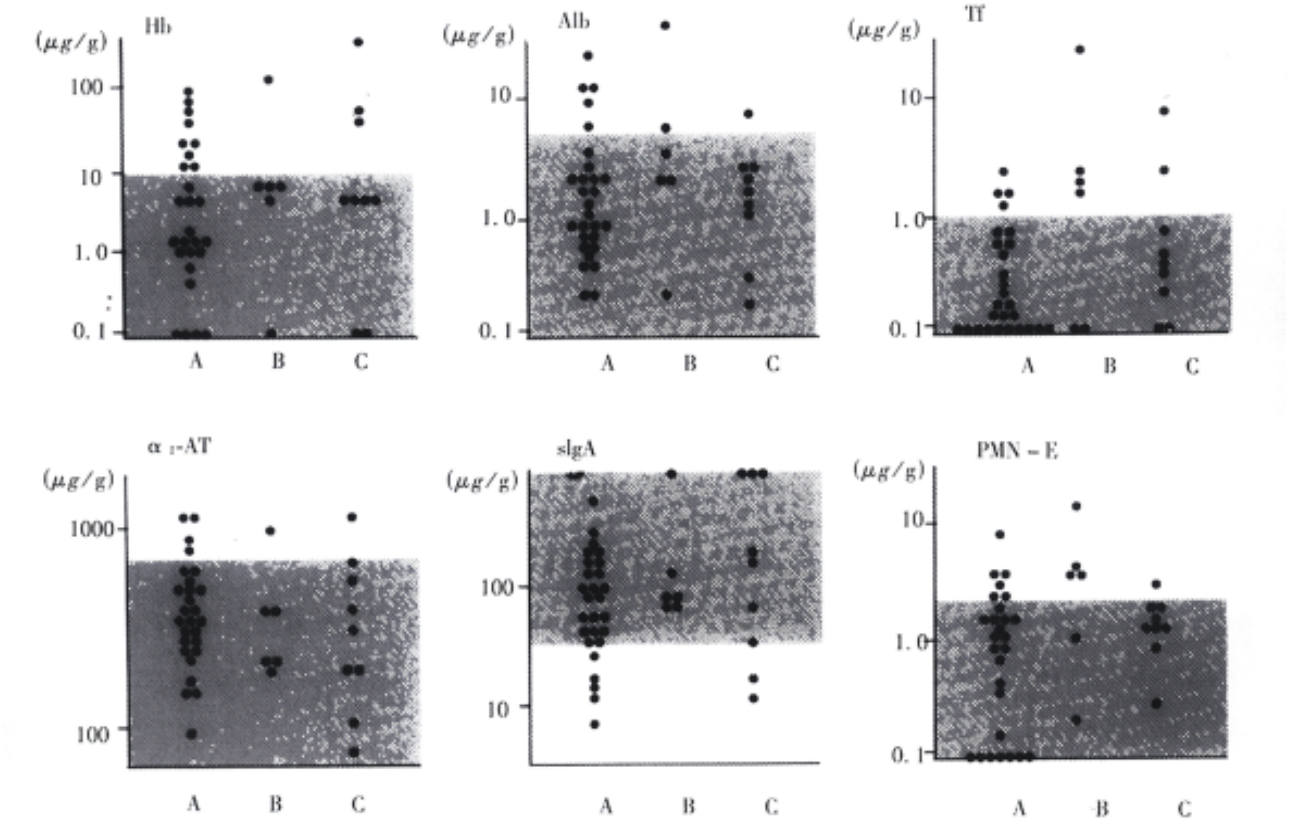

Figure2 Relationship between fecal protein concentr ations and the severity of the liver cirrhosis. A: Child A, B: Child B, C: Child C. The dotted area shows the 95 percentile of the control subjects.

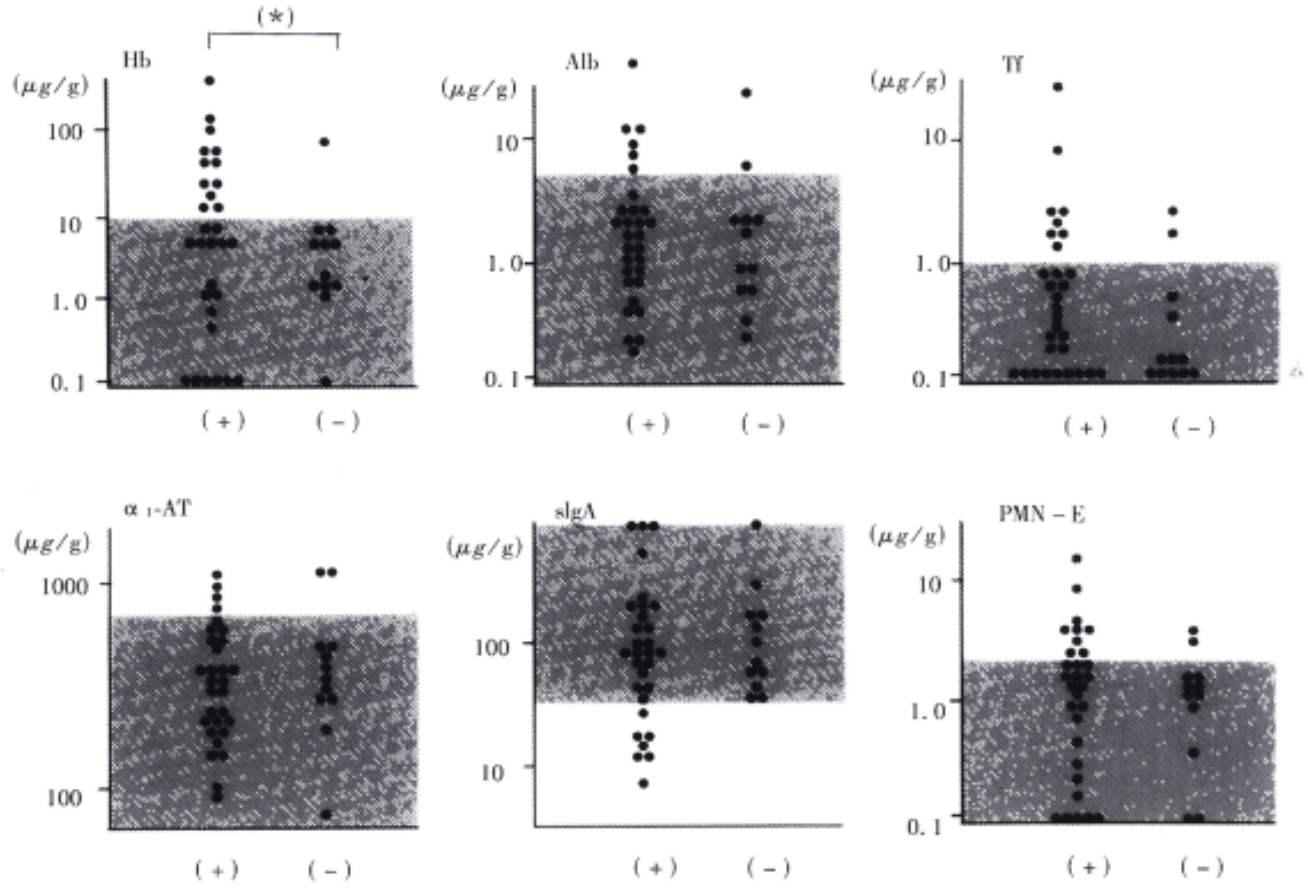

Figure3 Relationship between fecal protein concentr ations and the presence of esophageal varices. (+): cirrhotic patients associate d with esophageal varices; (-): cirrhotic patients not associated with esophagea l varices;[a]: $P<0.05$ by X^2 test.

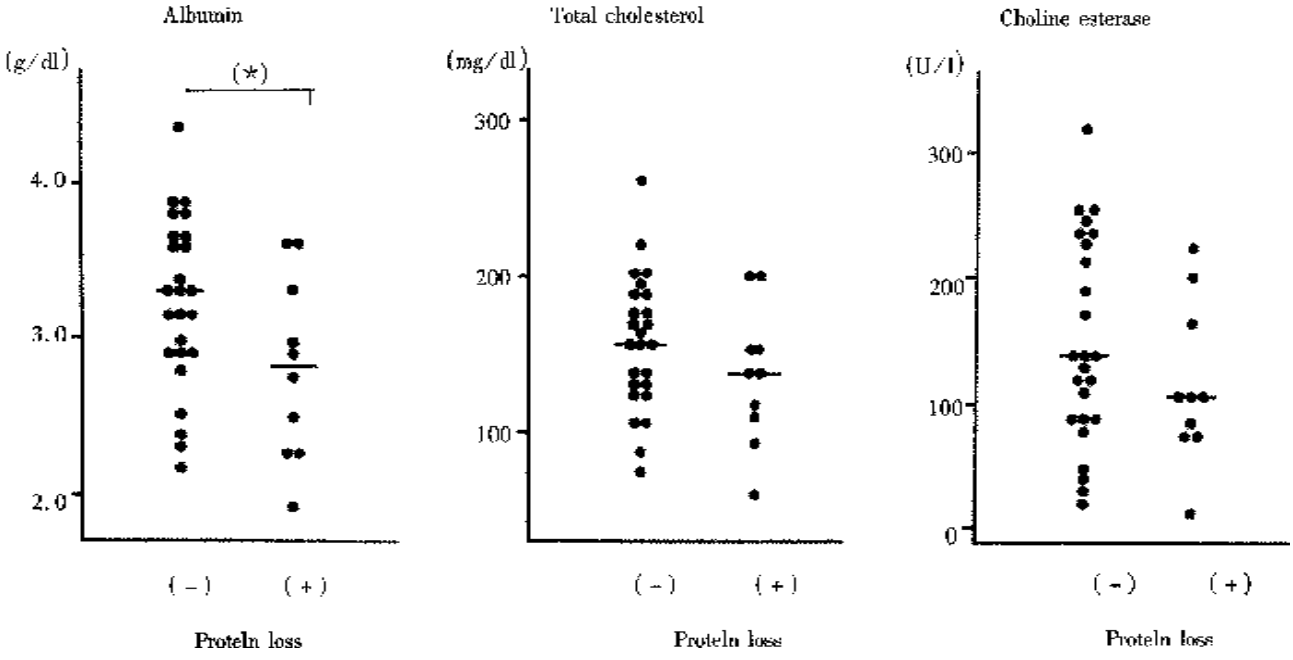

Figure4 Serum concentrations of albumin, total chol esterol, and choline esterase in cirrhotic patients. Comparison between groups with or without intestinal protein loss. [a]: $P<0.05$, NS; not significa nt by Mann-Whitney U test.

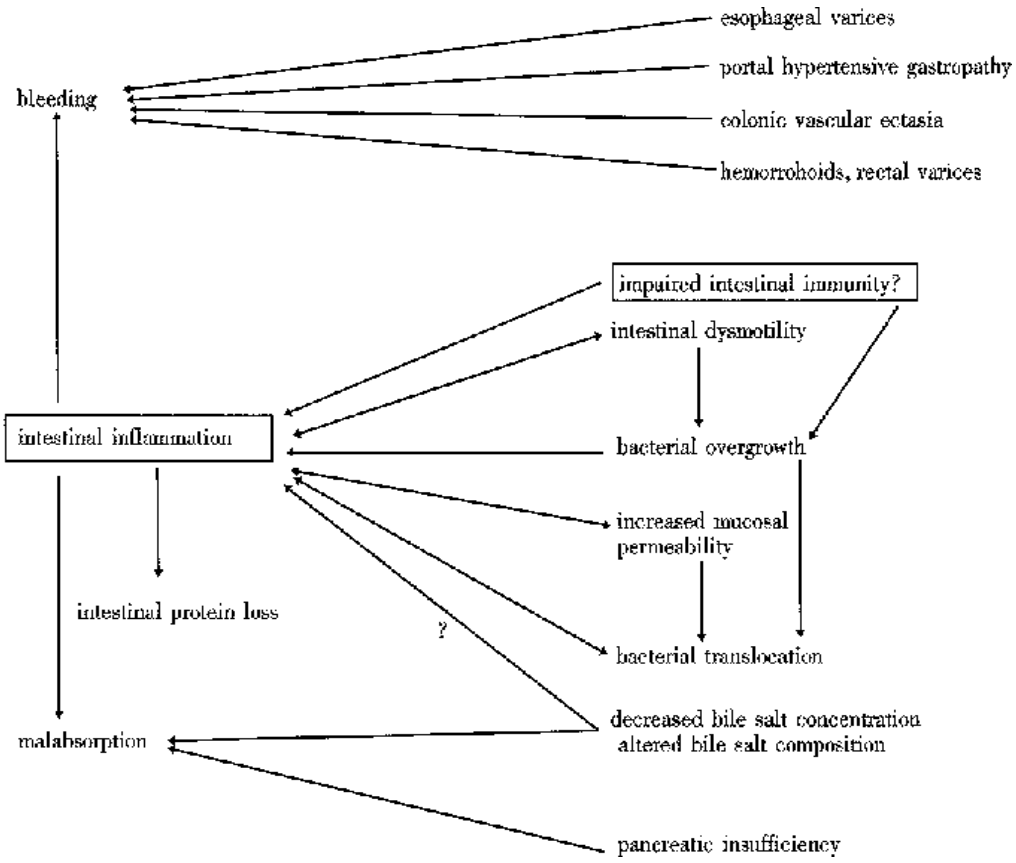

Figure5 Proposed pathophysiology of digestive tract in cirrhotic patients.

DISCUSSION

The present study showed that 26% of the cirrhotic patients had elevated fecal PMN-elastase concentrations and suggested an increased prevalence of intestinal inflammation in cirrhotic patients. Furthermore, occult GI bleeding and intestin alprotein loss were observed commonly even in cirrhotic patients who had no mas sive GI bleeding. The conditions such as bleeding, intestinal protein loss, impa ired intestinal immunity, and intestinal inflammation can be considered to be included in portal hypertensive enteropathy, because these seemed to be related not to the severity of the liver disease, but to portal hypertension. Recently, Stan ley *et al*[14] first demonstrated that many of the manifestations of portal hypertensive enteropathy can be corrected by a trasjugular intrahepatic portosystemic stent-shunt (TIPS) procedure. They described a cirrhotic patient with diarrhea and hypoalbuminemia. Protein losing enteropathy was confirmed by analysis of whole gut lavage fluid. They then performed a TIPS procedure, which eliminated the portal hypertension. Both the patient's diarrhea and elevated whole gut lavage fluid protein concentrations were reduced.

It is well known that the mechanism of hypoalbuminemia observed in cirrhotic patients is multifactorial. Decreased albumin production in the liver is one of the causes. In the present study, the serum Alb level was decreased significantly in patients with intestinal protein loss compared to that in patients without intestinal protein loss. The findings suggest that intestinal protein loss contributes to the development of hypoalbuminemia in cirrhotic patients.

Little attention has been paid to the relation between liver cirrhosis and intestinal inflammation. The prevalence of colitis diagnosed by colonoscopy in cirrhotic patients is not well established in the literature, and ranges from 10% to 57.9%[3,15-18]. Such a wide distribution is considered to be due to diff erences in the definition of inflammation and wide variation between observers in describing the mucosal appearance. The highest value, 57.9%, was reported by Scandalis N *et al*[16]. In their study, inflammatory changes included mucosal edema as well as erythema, granularity, and fragility of the mucosa. In contrast to colonoscopy, stool test is presumably a relatively objective test for assessment of inflammation. The value obtained in the present study (26%) was highest among the reported prevalence except for 57.9% reported by Scandalis N *et al*. The following possible reasons

should be taken into consideration. ① Fecal PMN-elastase, a marker of intestinal inflammation in the present study, reflects mucosal inflammation of the small intestine as well as the colon. The prevalence of inflammation of the small intestine has not been studied so far. ② Difference in sensitivity between the stool test and colonoscopy: the stool test seems to be more sensitive than colonoscopy, since colonoscopy does not always detect inflammation if the inflammation is minimal or localized.

Our proposed pathology of the digestive tract in cirrhotic patients is shown in Figure 5. The present study suggested that impaired intestinal immunity might be one of the predisposing factors to intestinal inflammation. In the present study, 17% of the cirrhotic patients showed decreased fecal sIgA concentrations. This is the first report concerning fecal sIgA in cirrhotic patients, although serum sIgA in cirrhotic patients was shown previously to be elevated[19]. Besides impaired intestinal immunity, predisposing factors for intestinal inflammation include intestinal dysmotility, bacterial overgrowth, bacterial translocation, and increased mucosal permeability[20]. Recently, we reported that bile acids inhibit tumor necrosis factor-α-induced interleukin-8 production in human intestinal cells[21]. A decreased level or an altered composition of the intraluminal bile acids may make cirrhotic patients suscept ible to intestinal inflammation[22,23].

It is well known that GI bleeding, intestinal protein loss, malabsorption, and bacterial translocation develop in patients with inflammatory bowel diseases[10,24,25]. In cirrhotic patients with intestinal inflammation, therefore, such serious pathological conditions may easily occur. Therefore, it is importa nt to evaluate whether a cirrhotic patient has intestinal inflammation or not in order to manage the patient. Measurement of fecal PMN-elastase is preferable to colonoscopy as a screening test for intestinal inflammation in cirrhotic patients, since the former is a sensitive and non-invasive test.

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Dynamic changes of type I, III and IV collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride-induced rat liver fibrosis

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See invited commmentary on page 388

Subject headings Procollagen mRNA; immunohistochemistry; Northern blot analysis; *in situ* hybridization; liver fibrosis

Abstract

AIM To find out the relationship between the gene transcripti on of different types of procollagen and the deposition of the relevant collagens in the liver tissue and to confirm the types of collagen producing cells in liver fibrogenesis.

METHODS Dynamic changes of the expression of α1(I), α1 (III) and α1(IV) procollagen mRNA and relevant collagens and the distribution of collagen producing cells during liver fibrogenesis of rat induced by CCl_4 (20 weeks) were investigated with Northern blot analysis, *in situ* hybrid ization and immunohistochemical techniques.

RESULTS The increased expression of α_1(III) procoll agen mRNA by Northern blot analysis was the most predominant one among the three mRNAs during fibrogenesis. However, the enhanced expression of α_1(IV) procollagen mRNA occurred very early while the expression of α1(I) mRNA was not enhanced much until the middle stage of the experiment. Desmin (Dm) positive hepatic stellate cells (HSCs) and few myofibroblasts (MFs) in and around the necrotic areas expressed α1(I), α1(III) and α1(IV) procollagen mRNA signals detected by *in situ* hybridization at the early stage of the experiment. All the three procollagen mRNA signals thereafter mainly localized in fibroblasts (Fbs) and MFs in fibrotic septa during the middle and late stages of fibrosis, which distributed parallel to the correspond ing collagens detected by immunohistochemical study. In addition, the endothelial cells of sinusoids and the small blood vessels within the septa also showed α1(IV) procollagen mRNA and type IV collagen expression

CONCLUSION It is considered that "HSC-MF-Fb" effect cell system is the major cellular source of collagen production in liver fibrosis, in which HSCs are collagen producing precursor cells in the early liver fibrogenesi s, thereafter the synthesis of type I, III and IV collagens (Col I, Col III and Col IV) mainly derives from MFs and Fbs, which play a very important role in the progress of liver fibrosis. The endothelial cells along sinusoids, as another s ource of Col IV production, might participate in the capillization of liver sinu soids.

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INTRODUCTION

The synthesis and the amount of collagen deposited in fibrotic liver have been studied by many investigators. The biochemical data revealed that the content of collagen proteins in liver fibrosis increased. Pierce *et al*[1] found that the increased total hydroxyproline was associated with collagen synthesis in early liver fibrosis of the rat induced by carbon tetrachloride (CCl_4). Oga wa *et al*[2] and Clement *et al*[3] investigated the localization and the semiquantitative analysis of the increased collagens in rat liver fibrosis immunohistochemically. The amount of α1(I), α1(III) and α1(IV) procollagen mRNA in liver fibrosis was found to be increased by either slot blot or Northern blot[4-6]. Many kinds of cells in liver fibrosis, such as hepat ic stellate cells (HSCs), myofibroblasts (MFs), fibroblasts (Fbs), parenchyma cells and endothelial cells were

reported to be involved in producing collagens in liver fibrosis *in vivo* and/or *in vitro*, but the results still remain controversial[1,4,6-12]. The application of immunohistochemical method pro vides a useful way for identifying the content and the components of the extrace llular matrix in the liver. However, it could not reliably identify the cell types which were responsible for synthesizing collagens during fibrogenesis. The *in situ* hybridization method provides a benefit to identifying the cell types expressing the extracellular matrix gene. Some studies demonstrated the α1(I), α1(III) and α1(IV) procollagen mRNA expressing cells with *in situ* hybridization using procollagen cRNA probes labeled with isotope ^{35}S *in vivo* and *in vitro*[7,8]. Because of the diffuse localization of ^{35}S-hybridization signals, it is also difficult to identify accurately the cellular composition signals which are exactly respons ible for the synthesis of collagen in fibrotic liver. The purpose of this study is to observe the dynamic changes of the expression of α1(I), α1(III) and α1(IV) procollagen mRNA by Northern blot analysis and the d istribution and content (semiquantitatively) of type I, III and IV collagen (Col I, Col III, Col IV) by immunohistochemistry in different stages of liver fibrog enesis and to clarify the types of collagen producing cells in liver fibrogenesi s by immunohistochemical staining of desmin (Dm) and á-smooth muscle actin (α-SMA) and in situ-hybridization of α1 (I), α1 (III) and α1(IV) procollagen mRNA with the procollagen cDNA probes labeled by digoxigenin in serial tissue sections.

MATERIALS AND METHODS

Animal model

Male Sprague-Dawley (SD) rats (n = 40, provided by Shanghai Division of the Animal Center of Chinese Academy of Sciences), weighing 180 g - 220 g, were subcutaneously injected with CCl_4 at a dose of 0.33 mL/100 g of body weight, and an equal mixture of olive oil twice a week with low-choline diet to induce liver fibrosis model[12]. The control rats (n = 24) were injected with an equal amount of olive oil only and fed with standard diet. Every five experimental rats were killed after 2-, 4-, 6-, 8-, 10-, 12-, 16-, and 20-week treatment and with 3 age-matched ones as control. After removal, small pieces of liver samples (5 mm^3-10 mm^3) were immediately froze n in liquid nitrogen and stored at -80 ℃ for Northern blot analysis, and other pieces of the liver tissues were cut into serial frozen sections with 5 μm in thickness and placed on poly-L-lysine coated slides. The sections were fixed in 4 mL/L paraformaldehyde in PBS (0.1 mol/L, pH 7.4, containing 5 mmol/L $MgCl_2$) for 10 minutes , rinsed in PBS, then in 2×SSC three times for 10 minutes each and dehydrated in ethanol and stored at -80 ℃ for *in situ* hybridization and immunohistochemistry.

Northern blot analysis

Rat α1(I), human α1(III) and α1(IV) procollagen cDNA plasmids were generously provided by Dr. Chu ML (Jefferson Medical College, USA). The sizes of the inserts contained in the pBR_{322} plasmid vectors are 1.3 kb for α1(I), 0.7 kb for α1(III) and 2.6 kb for α 1(IV) in size respectively. The cDNA probes were labeled with ^{32}P-deoxycytidine triphosphate (Amersham) to a specific activity of $(2.5) \times 10^8$ cpm/μg of DNA using a primer extension kit (Pharmacia) for α1(I) and α1(IV) procollagen cDNA probes labeling and nick translation kit (Gibco BRL) for α1(III) procollagen cDNA probe labeling. Total RNA was isolated from the liver tissue by extraction in guanidine isothiocynate[13]. Twenty μg total RNA from each sample was separated on a 10 g/L agarose gel containi ng 2.2 mol/L- formaldehyde and then transferred onto nitrocellulose filters (Stratagene), and baked at 80 ℃ for 2 hours to bind the RNA to the filters. The filters were prehybridized in 500 g/L formamide, 50 mol/L sodium phosphate, 0.8 mol/L NaCl, 1 mmol/L EDTA, and 2 g/L SDS in 4×Denhardt's solution with 250 μg/L denatured herring sperm DNA (Sigma) for 4 hours at 45 ℃ and then hybridized in the same fresh solution as above containing the ^{32}P-labeled α1(I), α1(III) and α1(IV) procollagen cDNA probes respectively overnight at 45 ℃. After high stringency washing in 2×SSC, 1×SSC, 0.5×SSC and 0.1×SSC with 1g/L SDS sequentially at 45 ℃ for 15 minutes each, the nitrocellulose filters were exposed to Kodak film at -80 ℃ for one week. After autoradiography, the filters w ere boiled in distilled water for 10 minutes to strip off the radioactive probes and rehybridized again with ^{32}P-labeled-glyceraldehyde-3-ph osphate dehydrogenase (GAPDH) cDNA probe (1×10^6 cpm/μg DNA) at 42 ℃ overnight for internal control. Autoradiographic signals of mRNA bands were quantified by scanning densitometry. The integrated optical density (IOD) of the hybridization bands were analyzed with TSTY-300 software (Sun Company of Tongji University). The IOD of α1(I), α1(III) and α1(IV) procollagen mRNA was corrected by the IOD of GAPDH mRNA. The data were analyzed by t test.

In situ hybridization

In situ hybridization was performed on poly-L-lysine-coated frozen tissue sections with slight modification of our previous report[14]. Briefly, frozen liver tissue sections (5 μm) were fixed in

40 mL/L paraformaldehyde before rehydrated with 0.1 mol/L PBS (pH 7.4), treated with 4 g/L Triton X-100 in PBS at RT for 10 minutes and incubated with proteinase K (20 mg/L, Merck Co.) in 0.1 mmol/L Tris at 37 ℃ for 30 minutes. Then the frozen tissue sections were postfixed with 40 mL/L p araformaldehyde in PBS (containing 5 mmol/L $MgCl_2$) again at RT for 10 minutes and quenched with glycine (2 m/L) in PBS for 5 minutes and acetyl ated in a freshly prepared solution of 2.5 mL/L acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0, Sigma) for 10 minutes. After rinsing with PBS, the tissue sections were dehydrated in graded ethanols and air dried prior to hybridization.

For *in situ* hybridization, the sections were prehybridized at 37 ℃ for 1 hour. Twenty μL of hybridization buffer was added to each section, followed by incubation in a sealed humid chamber at 42 ℃ for 16-18 hours. The hybridization buffer contained 500 mL/L deionized formamide, 100 g/L dextran sulfate (Sigma), 4×Denhardt's solution, 0.3 mol/L NaCl, 1 mmol/L EDTA (pH 8.0), 20 mmol/L Tris Cl (pH 8.0), 200 mg/L denatured herring sperm DNA (Sigma), 200 mg/L yeast tRNA (Sigma) and 500 μg/L denatured procollagen cDNA probes which were labeled with digoxigenin-dUTP by the random priming method using a DNA labeling and detection kit (Boehringer Mannheim). After hybridization the excess of the probe s was removed by rinsing in 2×SSC, 1×SSC and 0.5×SSC at 42 ℃ for 15 minutes each. The same kit as that used for DNA labeling was employed for immunological detection. The sections were washed briefly with buffer I solution (100 mmol/L Tris Cl, 150 mmol/L NaCl, pH 7.6), and incubated with buffer II solution which was prepared by buffer I solution with 10 g/L blocking re agent of the kit at RT for 30 minutes. After washing again briefly with buffer I solution, the sections were incubated with a 1:500-dilution of sheep a nti-digoxigenin Fab fragment conjugated with alkaline phosphatase in buffer II solution with 1 mmol/L levamisole (Sigma) at 37℃ for 2 hours. The sectio ns were washed twice with buffer I solution at RT for 15 minutes, and equilibrated with the buffer III solution (100 mmol/L TrisCl, 100 mmol/L NaCl, and 50 mmol/L $MgCl_2$, pH 9.5) for 10 minutes. Then the sections were incubated with the solution containing 4-nitroblue tetra zoliumchloride and 5-Bromo-4-chloro-3-indolyl-phosphate in buffer III to de velop the color in a dark box for 2 hours. After stopping the color reaction with buffer IV solution (10 mmol/L TrisCl, 1 mmol/L EDTA, pH8.0), the sections were mounted with glycerin and observed under light microscope.

Immunohistochemistry

The monoclonal antibodies against Col IV, α-SMA and Dm were purchased from DAKO. The rabbit anti-Col I, Col III and mouse and rabbit PAP kits were prepared by our department[15]. Col I, Col III, Col IV, α-S MA and Dm in normal and fibrotic livers were detected with PAP method as described previously[15]. Briefly, serial cryostat sections (5 μm) were treated with pure methanol (containing 0.2 mL/L H_2O_2) at 37 ℃ for 30 minutes and washed in PBS, and then incubated in PBS with 100 mL/L bovine albumin at 37 ℃ for 1 hour. The sections were incubated with mouse anti-type IV collagen (1:100 dilution), α SMA (1:40 dilution) and Dm (1:100 dilution), and rabbit anti-type I collagen (1:100 dilution) and type III collagen (1:1 000 dilution) respectively at 37 ℃ for 1 hour and then at 4 ℃ overnight. The sections were washed in PBS and incubated with rabbit anti-mouse (1:200 dilution) or goat anti-rabb it IgG (1:200 dilution) at 37 ℃ for 1 hour. After washing in PBS, the sections were incubated with mouse or rabbit PAP complex (1:200 dilution) at 37 ℃ for 1 hour. The color was developed with 0.5 g/L 3,3'-diaminobenzid ine/0.5 mL/L H_2O_2/0.05 mol/L TBS (pH 7.6) for 10 minutes. Normal mouse or rabbit serum instead of the specific primary antibodies were used as negative control.

RESULTS

Morphologic changes

Liver fibrosis model of the rats was successfully induced by CCl_4 subcutaneous injection with low-choline diet. At the 2nd week of the experiment, the hepato cytes of pericentral areas of lobules showed steatosis and necrosis. Likewise, HSCs proliferated and enlarged with enhanced Dm expression and some of them began to express α-SMA. At 4th-6th weeks of the experiment, fine cytofibrotic cords derived and extended from the periphery of the central veins. The cells in the cords were mainly composed of Dm and/or α-SMA positive MFs and the negative ones (Fbs) with long oval or spindle nuclei. There were also many activated Dm and α-SMA positive HSCs near the cords. In the middle stage of the experiment (8-12 weeks), the extended cytofibrotic septa connected the neighboring central areas or portal areas gradually. More Dm and/or α-SMA positive MFs or negative Fbs appeared within the septa also with Dm and α-SMA positive HSCs nearby (Figure 1). The proliferated oval cells in portal areas and septa expressed α-SMA and a few scattered hepatocytes might also express α-SMA. Newly formed capillaries could be found in the septa, and the long axes of which were parallel with the septa. In the late stage

(16-20 weeks), fibrotic septa was generally broadened. There were still many cells with long oval or spindle-shaped nuclei within the septa, however, most of them were both Dm and α-SMA negative. Only the cells located at the margin of the septa still kept positive staining. Daughter septa extended from the widened fibrotic septa might occur and cut the liver par enchyma further. The proliferated HSCs in the daughter septa continued to be α-SMA and Dm positive.

Procollagen mRNA Northern analysis

The expression of α1(I), α1(III) and α1(IV) procollag en mRNA was generally enhanced during the experiment, but the sequence and degree of the changes were not synchronous. At the 2nd week of the experiment, the content of -α1(IV)- procollagen mRNA was obviously increased compared with that of the normal. Then it decreased after the 4th week, but still kept a comparatively higher level during the whole period of the experiment. Although the enhancement of the expression of α1 (III) procollagen mRNA occurred later than that of α1(IV) procollagen mRNA, the increase of the expression of α1(III) procollagen mRNA was the most predominant one among the three in the whole experiment. The content of α1(III) procollagen mRNA reached its peak at the 10^{th} week, then decreased gradually. The expression of α1(I) procollagen mRNA was enhanced later than that of α1(III) mRNA and reached its first peak at the 12th week, then it decreased a little and increased again after the 16th week. Finally the conte nt of α1(I) procollagen mRNA reached the level as high as that of α1(III) procollagen mRNA at the 20th week (Figures 2, 3).

Procollagen mRNA in situ hybridization

The distribution of α1(I), α1(III), α1(IV) procollagen mRNA was similar in the normal rat liver, which mainly localized in mesothelial cells of the liver capsule, smooth muscle cells of blood vessels and periductal mesen chymal cells in portal areas, endothelial cells of central veins and a few HSCs in perisinusoids. In addition, α1(IV) procollagen mRNA transcription was also detected in sinusoid endothelial cells. In the early stage of the liver fibrogenesis there were a lot of stellate and spindle cells in the pericentra l areas of the lobules expressed strong signals of α1(III), α1(IV) procollagen mRNA and only some of them expressed α1(I) procollagen mRNA signals simultaneously. These cells were mainly regarded as the proliferated HSCs and MFs and proved to express Dm and/or α-SMA in the adjacent tissue sections. The cytofibrotic septa composed of HSCs, MFs and Fbs with positive hybridization signals connected the neighboring centrol areas and protal areas gradually (Figures 4, 5). The sinusoid endothelial cell s were also found to express strong signals of α1(IV) procollagen mRNA. In the middle stage of the experiment the MFs and Fbs in fibrotic septa further enhanced in expression of α1(III) procollagen mRNA, with some positive signals of α1 (I) and α1(IV) procollagen mRNA expression as well. In the late stage of the experiment the spindle-shaped cells within the septa often with negative staining of Dm and α-SMA expressed strong signals of α1(I) and α1(III) procollagen mRNA and weak signals of α1(IV) procollagen mRNA. In addition, the capillary endothelial cells of small blood vessels in the septa, some sinusoid endothelial cells and HSCs in perisinusoids also expressed strong α1(IV) procollagen mRNA (Figure 6). Hepatocytes, oval cells and the epithelia of bile ducts did not express any identifiable procollagen mRNA by *in situ* hybridization.

Immunohistochemical detection of collagens

Col I, Col III and Col IV of normal rat liver were mainly localized in liver capsule, portal areas and the walls of blood vessels. Liver sinusoids showed interruptedly positive staining with Col III and Col IV, but negative with Col I. In addition, Col IV was also localized at the basement membrane of capillaries and bile ducts. A few HSCs expressed type I and III procollagen with positive staining in cytoplasm. The change of the content of collagens in tissue sections of the experimental groups showed immunohistochemically the similar bias as that of procollagen mRNA by Northern analysis. In the early stage of the experiment, the content of Col III and Col IV was increased, which was mainly distributed in the necrotic areas or in fine cytofibrotic cards and was parallel to the distribution of the increased HSCs. The matrix of these areas was also stained with Col IV diffusely but weakly. However, the increase of Col I deposition was mild. In the middle stage, Col IV and Col III were further increased in the fibrotic septa. The decrease of α1(III) procollagen mRNA after the 10th week detected by Northern blot analysis did not seem to affect the content of Col III detected by immunohistochemistry, which was still steadily increased with fine fibers and diffusely distributed in and around the septa. Col I was also increased in the septa with thick fibers. Hyperplastic oval cells and cholangioli in the septa and hepatocytes did not express any procollagens. In the late stage, Col IV and Col III were decreased a little and the deposition of Col I was further increased in the broadened septa. The proliferated small blood vessels with positive staining of Col IV in basement membrane within the septa communicated with each other. The sinusoids were squeezed by the proliferated hepatocytes cords, most of which were Col IV and Col III positive continuously along the sinusoids.

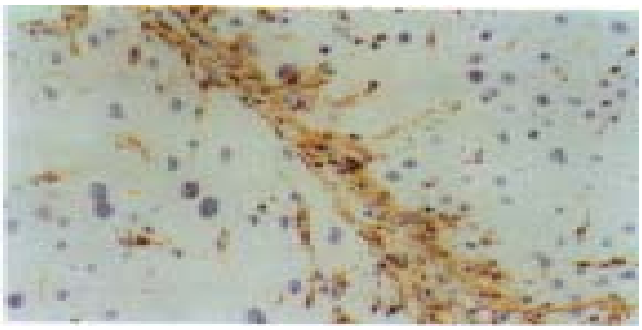

Figure1 The cells in the cytofibrotic septa we re mainly composed of desmin (Dm) positive hepatic stellate cells (HSCs) and myo fibroblasts (MFs) and some Dm negative fibroblasts (Fbs) (8 weeks of CCl_4 admi nistration). Immunohistochemical staining for Dm, ×200

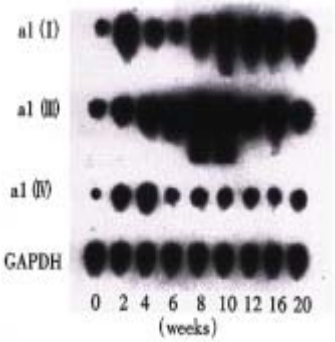

Figure2 Dynamic changes of α1 (I), α1 (III) and α1 (IV) procollagen mRNA expression in different stages of the experiment (Norther n blot analysis).

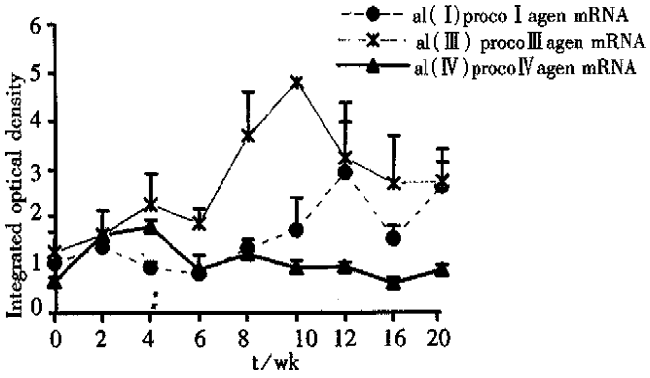

Figure3 The changes of α1 (I), α1 (III) and α1 (IV) procollagen mRNA expression in different stages of the experiment (Northern blot analysis).

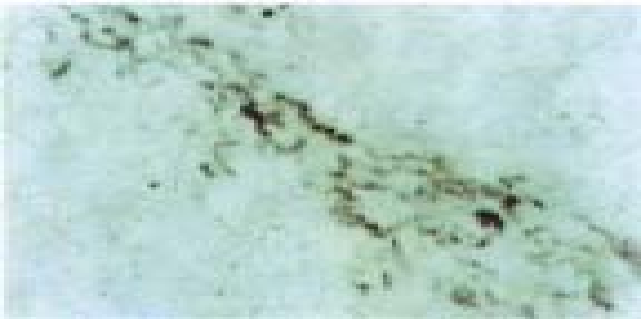

Figure4 Most of the cells in the cytofibrotic septa expressed α1(III)- procollagen mRNA (6 weeks of CCl_4 administration). *In situ*- hibridization,×400

Figure5 The serial tissue section of Figure 4, only a few of the cells in the cytofibrotic septa expressed α1(I) procollagen mRNA. *In situ* hybridization,×400

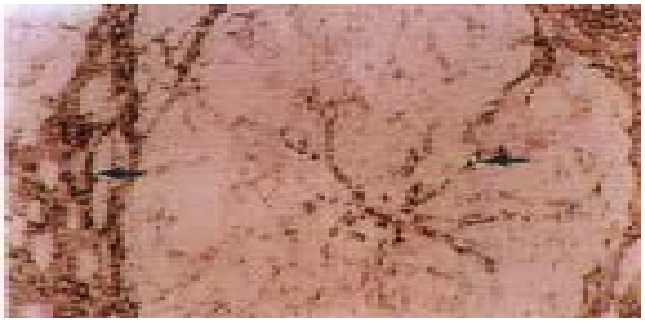

Figure6 Some spindle cells and the endothelial cell s of small blood (arrow) vessels in the fibrotic septa and the endothelial cells of the capillized sinusoids (arrow) expressed strong signals of á1 (IV) procoll agen mRNA in the late stage of the experiment (16 weeks of CCl_4 administration). *In situ* hybridization, ×200

DISCUSSION

The fibrogenic factors known to contribute to liver fibrosis included CCl_4 administration, alcohol intake, dimethylnitrosamine administration, bile duct obstruction, iron or cholesterol overload, low-nutrient diet, schistosomiasis, immune complex induction, hepatitis virus, etc[1,4-6,16-18]. The gene expression of procollagen in liver fibrosis was predominant. The increased procollagen mRNA levels in liver fibrosis might result from both the increased gene transcription and increased mRNA stabilization. In fact, both of the mechanisms have been demonstrated to increase procollagen mRNA levels *in vivo* and *in vitro*[1,6-8]. Furthermore, some of cytokines might also be involved in the enhancement of expression of procollagen mRNA[5,19-21]. In the present st udy, the content of α1(I), α1(III) and α1(IV) procollagen mRNA expression in the liver detected by Northern blot analysis are all changed drama tically, but not synchronously during fibrogenesis. In the early stage of the ex periment, the expression of α1(IV) procollagen mRNA was increased first, then that of α1(III) mRNA, which always kept the predominant expression over the other two. The expression of the α1(I) procollagen gene increased slowly during

the early stage, not as high as the content of α1(III) mRNA until the 20th week. It suggests that there is some difference in the regulation of transcription and stabilization of these procollagen genes. The data from early liver fibrosis in this study were similar to those of Nakatsukasa *et al*[8] and our previous investigat ion[14], but a bit different from that of Pierce *et al*[1], in which slot blot analysis showed the expression of α 1(I) procoll agen mRNA was enhanced at a slightly earlier point in the liver fibrogenesis than those of α1(III), and α1(IV) procollagen mRNA. The reason for the discrepancy between the studies is not clear. It might attribute to different ways of CCl_4 administration; involvement of other fibrogenic factors; and different methods, probes or animals used, etc.

Gene expression control is a very precise and complex mechanism, which is involved at transcription and post-transcription levels. It was reported that the pol ypeptide fragment of type I collagen could inhibit the transcription and transl ation of α1(I) procollagen gene, and result in a feedback inhibiti on in procollagen polypeptide synthesis[22-24]. Perhaps it is because either the activation of procollagen gene or the feedback inhibition exerted by c ollagen polypeptides acted alternately so that the enhancement of the procollagen gene transcription during fibrogenesis revealed a ladder-shaped curve rather than a straight-line one. Moreover, the changes of collagen protein contents (average grade detected immunohistochemically) in liver during fibrogenesis were not consistant with the expression contents of their relevant procollagen mRNA completely, possibly because the content of procollagen mRNA was closely related to the real production of procollagen at the certain moment of the experiment, whereas the content of collagen protein in fibrotic liver revealed by immunohistochemistry in fact depended on all the finally accumulated content of collagen p roduction, deposition, and degradation in liver tissue[14,25-27]. For this reason, the procollagen gene detection by nucleic hybridization can more sen sitively and objectively reflect the trend of collagen synthesis in liver fibrog enesis.

Much more researches have focused on the study of the source of collagen-produc ing cells in liver fibrosis[4-8,10,14,16]. Besides HSCs and the related MFs, Fbs, whether any other cells, i.e. hepatocytes, endothelial cells and bile duct epithelial cell involved in the collagen production, is under active invest igation. Some authors indicated that the collagens *in vivo* produced by hepatocytes both in normal and fibrotic liver was responsible for the principal amount of collagen content in liver[3,28]. However, the data from other inves tigators demonstrated that hepatocytes had no collagen-synthesizing ability [7]. *In vitro*, several authors reported that the cultured hepatocytes could synthesize collagens spontaneously. But from the other investigators' point of view, the reason why the collagens appeared in cultured hepatocytes and endothelial cells was because of HSCs contamination[29,30]. Recent studies s howed that HSCs, when stimulated by fibrogenic factors, were activated and enhan ced in expression of Dm and α-SMA, and underwent a phenotypic transformation to MFs and Fbs, around which there was the deposition of collagens. T he increase of the number of these cells was parallel to the increase of the content of collagen deposition and to the changes of their distribution in the liver. Therefore, we suggested that there might be a "HSC-MF-Fb" effect cell sys tem in liver fibrosis, which was responsible for collagen synthesis[12]. However, the conclusion described above was mainly derived from the results of our previous immunohistochemical and ultrastructural studies. Up to now, there are still contraversies regarding which cell population offers the major contribu tion to liver fibrosis. In this study, the content and the distribution of α1(I), α1(III), and α1(IV) proco llagen mRNA and the relevant collagens of rat liver fibrosis and cirrhosis were observed in detail by both immuno histochemistry and *in situ* hybridization with the procollagen cDNA probes la beled with digoxingenin. The role of "HSC-MF-Fb" effect cell system of colla gen synthesis during liver fibrogenesis was further explored. In the early stage of the experiment, a variety of Dm and/or α-SMA positive HSCs a nd MFs appeared in necrotic areas around central veins enhanced in the expression of α1(III), α1(IV) and α1(I) procollagen mRNA.

Endothelial cells of sinusoids also increased in the expression of α1(IV) procollagen mRNA. The distribution of α1(I), α1(III) and α1(IV) procollagen mRNA expression cells was similar to those of the deposition of the corresponding collagens. At the middle stage of the experiment, the hybridization signals of these procollagen mRNA were mainly localized in the Dm and/or α-SMA positive MFs within fibrotic septa and the HSCs around the septa. During the late stage of liver fibrogenesis, except for newly formed daughter septa, the number of Dm and/or α-SMA positive cells in the fibrotic septa decreased gradually. However, the hybridization signals of these procollagen mRNA was still numerous in the cells with long oval or spindle-shaped nuclei within the septa. Therefore, the hybridiz ation signals of the procollagen mRNA in well developed fibrotic septa was mainly derived from Fbs. Our results are consistent with those of several investigators who

demonstrated that HSCs, as well as MFs and Fbs, contributed to the major source of collagen synthesis during liver fibrogenesis by either *in situ* hybridization or immunohistochemically[7,8]. In contrast, no evidence of collagen synthesis by hyperplastic hepatocytes and chlangioli-like tubules, wh ich embedded in the increased extracellular matrix of fibrotic septa and often with large amount of Fbs nearby, could be observed both *in situ* hybridization and immunohistochemically in the present study.

On the basis of our findings described above, it is confirmed that "HSC-MF-Fb" effect cell system is the major cell source of collagen synthesis during live r fibrogenesis, in which HSCs are the collagen-producing precursor cells in the liver. In and after the middle stage of liver fibrosis, the production of Col I, Col III, and Col IV in the fibrotic septa are mainly derived from both MFs an d Fbs. The expression of procollagen mRNA in these cells plays a very important role in the process of liver fibrosis. Furthermore, sinusoid endothelial cells, as another source of Col IV production, might also participate in the capillizat ion of liver sinusoids.

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Inhibitory activity of polysaccharide extracts from three kinds of edible fungi on proliferation of human hepatoma SMMC-7721 cell and mouse implanted S180 tumor

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Subject headings polysaccharide, edible fungi; liver neoplasm; carcinoma, hepatocellular; SMMC-7721, tumor cell, cultured; implanted tumor, S-180; cell proliferation

Abstract

AIM To determine the activities of polysaccharide extracts from *Flammulina velutipes* (Curt. ex Fr.) Sing (FV), Lentinus edodes (LE) and Agaricus bisporus-Sing (*AB*) on the proliferation of human hepat oma SMMC-7721 cells *in vitro* and on mouse implanted S-180 tumors *in vivo*.

METHODS The polysaccharide extracts were isolated from the fru it bodies of FV, LE and *AB* by the methods of hot-water extraction, Sevag's removal of proteins, ethanol precipitation, trypsin digestion and ethanol fractional precipitation. Human hepatoma SMMC-7721 cells were treated with 50mg/L polysaccharide extracts, and the mitosis index, mitochondria activity and cell proliferation were detected at different times in both control and experimental groups. The mice with S-180 implanted tumors were injected with the polysaccharide extracts at 24mg/kg body weight for 9d and the tumor weight was measured on the 15th day.

RESULTS The mitosis index of hepatoma cells *in vitro* could be significantly decreased by treatment with the polysaccharide extracts from the three kinds of edible fungi ($P<0.005$). The cell numbers and mitochondria activity of SMMC-7721 cells treated with polysaccharide extracts were lower than those in control groups ($P<0.005$). The inhibition rates of polysaccharide extracts against implanted S-180 tumors in mice were 52.8%, 56.6% and 51. 9% respectively compared with that in control groups.

CONCLUSION The polysaccharide extracts from the three kinds o f edible fungi could inhibit not only the cultured malignant cells *in vitro* but also implanted S-180 tumor *in vivo*.

INTRODUCTION

The polysaccharides from edible fungi (e.g. *LE*[1], *FV*[2,3]) were macromolecular substances with strong antigenicity and were also verified to have antitumor activity against S-180 implanted tumor in mice *in vivo* and that from *AB* were shown to have anti-infection of virus and anticanceration *in vivo*. The references about the effects of polysaccharide extracts from edible fungi on cancer cells *in vitro* were very limited. In this report human hepatoma SMMC-7721 cells were used as a model to detect the anticancer activity of polysaccharide extracts from the three kinds of edible fungi (*FV, LE* and *AB*). The mitosis index, cell proliferation and mitochondria metabolism a ctivity of SMMC-7721 cells were compared between the control group and polysacc haride extracts treatment groups. The antitumor activity of polysaccharide extracts from these three kinds of edible fungi against implanted S-180 tumor in mice *in vivo* was also observed.

MATERIALS AND METHODS

RPMI 1640 medium is product of GIBCO; trypsin and MTT were from Sigma; the fruit bodies of *FV, LE* and *AB* were from cultivated products in Jinan. Mice with S-180 and Kunming male mice (22 g - 25 g) were from Shandong Experiment al Animal Center; 24 and 96-well plates were from Costar.

Cell lines and culturing

Human hepatoma SMMC-7721 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in our laboratory. The cells were grown as monolayers in RPMI 1640

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medium supplemented with 10% fetal calf serum (FCS) and incubated at 37 ℃ in the humidified incubator with 5% CO_2/95% air.

Extraction and purification of polysaccharide extracts from the edible fungi

The extraction and purification of polysaccharide extracts were modified according to the methods of Cao[3]. The fresh fruit bodies of the edible fungi were homogenized for three times, heated at 98 ℃-100 ℃ for 5 h, centrifuged for 15 min with 4500 rpm. The supernatants were collected and the precipitation was extracted for another two times. All the supernatants were concentrated to proper volume and precipitated with 3 times 95% ethanol and stayed overnight at 4 ℃. The precipitation was gathered by centrifugation, dissolved in distilled water, dialyzed in 4 ℃ distilled water for 2 d, digested with trypsin and precipitated protein with Sevag's method. The polysaccharide extracts were dialyzed in 4 ℃ distilled water for another 2 d, precipitated with 3 times 95% ethanol, stayed overnight and collected with centrifuge. The white precipitation of polysaccharide extracts from the three edible fungi was rinsed with 100% ethanol and acetone and dried at room temperature.

Determination of polysaccharide extracts on the metabolism of mitochondria

The exponent growing SMMC-7721 cells in culture flasks were harvested by trypsinization with 0.25% trypsin, suspended in RPMI 1640 medium with 10% FCS, adjust ed to the concentration of 1×10^5 cells/mL, plated into 96-well plates (200 μL cells/well) and incubated at 37 ℃ in 5% CO_2/95% air for 24 h. The medium was aspirated and the cells were washed with RPMI 1640 medium. The medium was replaced with RPMI 1640 containing 50 mg/L polysaccharide extracts from different fungi as treatment groups respectively and medium with 10% FCS and Non-FCS as controls (each group having 8 repeated wells). The cells were incubated in different treatments for 20 h, 44 h and 68 h respectively. The metabolism of mitochondria[4] were detected by adding 20 μL- MTT (final concentration 10 mg/L) to media, incubating for 4 h, sucking out the media, adding 100 μL dimethylsulfoxide (DMSO) to dissolve the violet-crystal and measuring the absorption at 570 nm.

Measurement of mitosis index

The exponent growing SMMC-7721 cells were suspended in RPMI 1640 medium plus 10 % FCS, adjusted to 1×10^5 cells/mL, plated 2 mL cells/well into 24-well plates (with cover glass, 4 repeated wells in each group) and incubated at 37 ℃ for 24 h. After incubated in medium containing different polysaccharide ext racts (experiment groups) and 10% FCS or non-FCS (as control groups) for 24 h, the cells were fixed with Carnoy solution and stained with Feulgen reaction. The mitosis indexes were detected randomly by counting 1000 cells[5].

Proliferation of SMMC-7721 cells in medium containing polysaccharide extracts

The SMMC-7721 cells were treated with different polysaccharide extracts for 7 d as above (6 repeated wells in each group) and the proliferation of SMMC-7721 cells were observed by counting the cell number every day.

In vivo experiments with implanted S-180 tumor

The S-180 tumor cells were washed with normal physiological saline for three times, adjusted to 1×10^7 cells/mL and implanted by subcutaneous injection 200 μL to each mouse. Twenty-four hours late, the mice were injectedip with 24 mg polysaccharide extracts/kg body weight in experimental groups and physiological saline in control group for 9 d. On the 15th day after the treatment, the mice were killed and the tumors were isolated a nd weighed. The inhibition rate of tumor was calculated as follows:

$$\frac{\text{(Mean tumor weight in controls-mean tumor weight in experiments)}}{\text{Mean tumor weight in control group}}\times100\%$$

RESULTS

The production of polysaccharide extracts

The production of the polysaccharide extracts extracted from the three kinds of edible fungi were 1.53 mg/g ± 0.11 mg/g fresh fruit bodies of *LE*; 4mg/g ± 0.15 mg/g fresh fruit bodies of *FV* and 1.3 mg/g ± 0.11 mg/g fresh fruit bodies of *AB*, respectively. None of the polysaccharide extracts showed obvious absorption in the range of 220 nm-780 nm.

The effects of polysaccharide extracts on the metabolism of mitochondria

Within 24 h, the mitochondria metabolism of hepatoma SMMC-7721 cells showed no obvious differences in each group. At 48 h and 72 h, the metabolism activities in experimental groups were much lower than those in both FCS and non-FCS control groups, but in different experimental groups they were similar (Figure 1).

The effects of polysaccharide extracts on mitosis index (MI)

The MI in all the groups treated with polysaccharide extracts was lower than that in both control groups (P<0.005) (Table 1).

The effects of polysaccharide extracts on the proliferation of SM MC-7721 cells

The cell number after treatment with polysaccharide extracts for 48 h were obviously lower as compared with both FCS and non-FCS controls (Figure 2). On the 7th day the number of the cells covered 55.5% (*LE*), 59.5% (*FV*) and 61.6% (*AB*) of that in the FCS control and 63% (*LE*), 67.6% (*FV*) and 70. 3% (*AB*) of that in non-FCS control.

The inhibition of polysaccharide extracts on the proliferation of S-1 80 implanted tumor

The inhibitions of the three polysaccharide extracts on the proliferation of the S-180 tumor *in vivo* were 52.8% (*LE*), 56.6% (*FV*) and 51.9% (*AB*), respectively (Table 2).

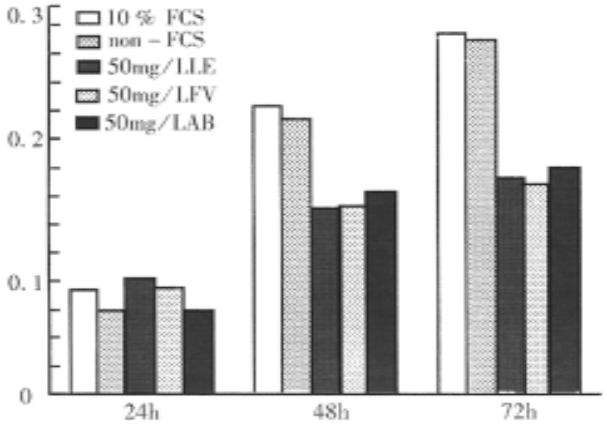

Figure1 Effects of polysaccharide extracts on the m itochondria metabolism of SMMC-7721 cells.

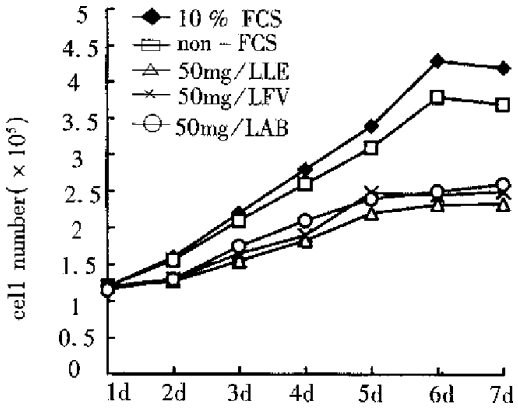

Figure2 The effects of polysaccharide extracts on the proliferation of SMMC-7721 cells.

Table 1 The effects of polysaccharide extracts on the MI of SMMC-7721 cells

Groups	MI(%)	*P* value
C1	18.2±1.1	
C2	13.4±1.2	
E1	5.8±0.7	<0.005
E2	8.5±0.9	<0.005
E3	8.4±0.8	<0.005

C1: 10% FCS; C2: non-FCS; E1: 50 mg/L polysaccharide extracts from *LE*; E2: 50 mg/L polysaccharide extracts from *FV*; E3: 50 mg/L polysaccharide extracts from *AB*.

Table 2 The inhibitions of polysaccharide extracts on the proliferat ion of S-180 implanted tumor *in vivo*

Groups	*n*	Dose (mg/kg)	Tumor weight (g)	Inhibition (%)	*P* value	Mean inhibition (%)
LE 1	10	24	0.369±0.058	55.5	<0.005	52.8
LE 2	10	24	0.387±0.069	53.3	<0.005	
LE 3	10	24	0.417±0.085	49.7	<0.005	
FV 1	10	24	0.371±0.034	55.3	<0.005	56.6
FV 2	10	24	0.348±0.039	58.1	<0.005	
FV 3	10	24	0.376±0.044	54.7	<0.005	
AB 1	10	24	0.379±0.049	54.3	<0.005	51.9
AB 2	10	24	0.39±0.067	53	<0.005	
AB 3	10	24	0.427±0.087	48.5	<0.005	
Control	20		0.83±0.17			

DISCUSSION

The efficiency of polysaccharide extracts

Following the same processes, the products of polysaccharide extracts in the three kinds of edible fungi were obviously different. The product of polysaccharide extracts in *FV* was 4 mg/g fresh fruit body, which was 4 times higher than that in *AB* and 2.6 times than in *LE*. These differences should be induced by the contents of polysaccharide in different kinds of edible fungi. Although the polysaccharide extracts were a mixture of polysaccharides, they had no obvious absorption peak in the range of 220 nm-780 nm.

The effects of polysaccharide extracts on the hepatoma cell in vitro and S-180 in vivo

The polysaccharide from *LE* was the first extracts which verified the anti-tumor activity in 1969[1], which initiated the study of extraction, purification, structure analysis and anti-tumor activity of polysaccharides from *LE*. The best result of anti-tumor activity of polysaccharides from *LE* could reach 90%-100% *in vivo*. So polysaccharides from *LE* were believed to be one of the best effective substances for antitumor treatment. On the other hand the polysaccharide from *LE* was also verified to have anti-mutant activity[6]. Both pure and mixed polysaccharides from *FV* were shown to have strong anti-tumor activity against implanted S-180 tumor in mice[3,7] . *In vivo* the anti-tumor activities of polysaccharides from edible fungi were mainly induced by activating the immune system, although the detailed mechanism was not clear. In this study, the cultured hepatoma SMMC-7721 cells were used as a model to detect the activity of polysaccharide extracts *in vitro*. The results of MI, mitochondria activity and cell proliferation showed that the extracts from the edible fungi could inhibit the division of the cells and induce the decrease of cell proliferation, which

was similar to that from fruit bodies of Ganoderma lucidum[8]. The inhibition of the extracts from the three kinds of edible fungi on the growth of implanted S-180 tumor *in vivo* could reach 52.8%, 56.6% and 51.9% respectively, which agreed with the previous reports[3].

These results suggested that the polysaccharide extracts from *LE*, *FV* and *AB* could not only inhibit the growth of implanted S-180 tumor *in vivo* but interfere with the proliferation of human hepatoma SMMC-7721 cells *in vitro* .

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Regulatory effect and mechanism of gastrin and its antagonists on colorectal carcinoma

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Subject headings colorectal neoplasms; gastrin; proglumide; somatostatin; IP_3, Ca^{2+}; protein kinase C; oncogene; AgNORs

Abstract

AIM To explore the effect and mechanism of gastrin and its an tagonists proglumide and somatostatin on colorectal carcinoma and their clinical significance.

METHODS A model of transplanted human colonic carcinoma was established from SW480 cell line in gymnomouse body. The volume and weight of trans planted carcinoma was observed under the effect of pentagatrin (PG), proglumide (PGL) and octapeptide somotostatin (SMS201-995, SMS). The cAMP content of carci noma cell was determined by radioimmunoassay and the DNA, protein content and cell cycle were determined by flow-cytometry. The amount of viable cells was dete rmined by MTT colorimetric analysis, IP_3 content was determined by radioimmuno assay, Ca^{2+} concentration in cell by fluorometry and PKC activity by isotopic enzymolysis. The expression of gastrin, c-myc, c-fos and rasP21 in 48 case s of colorectal carcinoma tissue was detected by the immuno-cytochemistry SP method. Argyrophilia nucleolar organizer regions was determined with argyrophilia stain.

RESULTS The volume, weight, cAMP, DNA and protein content in carcinoma cell, cell amount and proliferation index of S and G_2M phase in PG group were all significantly higher than those of control group. When PG was at the concentration of 25 mg/L, the amount of viable cells, IP_3 content and C a^{2+} concentration in cell and membrane PKC activity in PG group were signi ficantly higher than those in control group; when PGL was at a concentration of 32 mg/L, they dropped to the lowest level in PG (25 mg/L) +PGL group, but without significant difference from the control group. The positive expression rate of gastrin, c-myc, c-fos and rasP21 in carcinoma tissue was 39.6%, 54.2%, 47.9% and 54.2% respectively and significantly higher than that in mucos a 3 cm and 6cm adjacent to carcinoma tissue and normal colorectal mucosa. The pos itive expression rate of gastrin of highly-differentiated adenocarcinoma group was significantly higher than that of poorly-differentiated and mucinous adenoc arcinoma groups. The AgNORs count of carcinoma tissue was significantly higher than that in mucosa 3 cm and 6 cm adjacent to carcinoma tissue and norm al colorectal mucosa; and the positive expression of c-myc and c-fos and the A gNORs count in gastrin-positive group was significantly higher than those in gastrin-negative group.

CONCLUSION Pentagastrin has a promoting effect on the growth of transplanted human colonic carcinoma from SW480 cell line. PGL has no obvious effect on the growth of human colonic carcinoma SW480 cell line, but could inhibi t the growth promoting effect of PG on transplanted carcinoma. Somatostatin can not only inhibit the growth of transplanted human colonic carcinoma from SW480 cell line directly but also depress the growth-promoting effect of gastrin on t he transplanted carcinoma. Some colorectal carcinoma cells can produce and secrete gastrin through autocrine, highly-differentiated adenocarcinoma express the highest level gastrin. Endogenous gastrin can stimulate the cell division and pr oliferation of carcinoma cell and promote the growth of colorectal carcinoma regulating the expression of oncogene c-myc, c-fos. Our study has provided experi mental basis for the adjuvant treatment using gastrin antagonist such as PGL, so matostatin of patients with colorectal carcinoma.

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INTRODUCTION

In recent years, some studies indicated that gastrin could promote the growth of some colorectal carcinomas, but gastrin antagonist such as PGL, somatostatin (SS) could inhibit the growth of those colorectal carcinomas. In order to explore the mechanism of the effect and the clinical significance of gastrin and its antagonists on colorectal carcinoma, we established the model of transplanted colonic carcinoma from SW480 cell line in gymnomouse body, and observed the volume and weight of transplanted carcinoma, content of cAMP, DNA, protein and cell cycle of carcinoma cell under the effect of pentagastrin (PG), proglumide (PGL) and o ctapeptide somatostatin (SMS 201-995, SMS). We also observed the effect of PG and PGL on the amount of viable cells, inositol 1, 4, 5-trisphosphate (IP_3) content and Ca^{2+} concentration in cell and protein kinase C (PKC) activity of colonic carcinoma SW480 cell line cultured *in vitro*, and detected the expression of gastrin, cancer genes c-myc, c-fos and rasP21 in 48 cases of colorect al carcinoma tissue by the immunocytochemical SP method and determined the argyr ophilia nucleolar organizer regions count with argyrophilia stain.

MATERIALS AND METHODS

Materials

Human colonic carcinoma SW480 cell line (Sloan Kittin Memorial Cancer Center, US A), BALB/C gymnomouse (Experimental Animal Center of Third Military Medical Univ ersity), pentagastrin (PG, Shanghai Lizhu Chemical Reagent Co.), octapeptide som atostatin (SMS 201-995, SMS, PHARMA, Swiss); proglumide (PGL, No.11 Pharmacenti cal Factory, Shanghai), RPMI1640 culture solution (GIBCO, USA), 3H-myo-inositol (Institute of Atomic Energy, Chinese Academy of Sciences), T-^{32}P -ATP (Ya Hui Biology Engineer Co.), Fura-2/ AM (Sigma, USA), cAMP radioimmu noassay reagent kit (Department of Nuclear Medicine of Shanghai Second Medical University), multiclonal antibody to gastrin and SP kit (ZYMED Co., USA), monoclo nal antibody for c-myc and rasP21, multiclonal antibody for c-fos (Santa Cruz Co., USA).

Experiment methods

Establishment of transplanted carcinoma model The human colonic carcinoma SW480 cell line was resuccitated conventionally. Cell lines with exub erant vitality were selected to form primary transplanted carcinoma, digested with 0.25% trypsin, centrifugated to wipe off the digestive solution, and adjust ed the cell concentration with RPMI1640 cultural solution to 5×10^6/mL. The living cell amount exceeded 99% by trypan-blue stain. Twelve gymnomice were ran domized into six groups. The cell line went through six passages, each time two gymnomice were inoculated. After 0.2 mL of carcinoma cell solution was inoculated to the back of the neck of gymnomouse, the gymnomice were raised in clean room. When the diameter of the carcinoma reached 1.5 cm to 2.2 cm, the gymnomice were killed by severing the cervical vertebra, and the carcinoma mass was stipped bacteria-freely. After preserving specimen for histological and electron microscopical examination, the mass was smashed, ground into cell suspension with RPIM1640 cultural solution, adjusted the cell concentration to 5×10^6/mL, the next passage was started with 0.2 mL of the cell suspension.

Experimental animal grouping After undergoing passages stably-growing human colonic transplanted carcinoma cells were inoculated into thirty gymnomice. They were randomized into six groups and injected with the experimental drugs subcutaneously the next day, two times per day, for 35 days. The gymnomice were killed 24 hours after the last injection. Control group: 0.4 mL normal saline/mouse. PG group: 4 μg PG/ mouse. PGL group: 10 mg PGL/mouse. SMS group: 6 μg SMS/ mouse. PG+PGL group: 4 μg PG and 10 mg PGL/ mouse. PG+SMS group: 4 μ PG and 6 μg SMS/ mouse.

Volume and weight of transplanted carcinoma The long diameter and short diameter were measured at the same time every sixth days after the inoculation, and the measurement lasted six weeks. The volume was calculated by the formula: $V = 1/2a^2b$. The weight of the mass was then determined after decontamination of the non tumor tissue such as blood and fat tissue.

Flow-cytometry Fifteen g transplanted carcinoma tissue was cut and made into single-cell suspension[1], adjusted to a cell concentration of 1×10^8/L, and fixed with 700 g/L alcohol at 4 ℃. The DNA was stained with propidium iodide to redish flourescence and the protein stained with fluorescein iso thiocyanate to green flourescence. Thirty minutes later, the suspension was analyzed on instrument at room temperature. According to Barlogie cell cycle analysis method, the cells were divided into three parts: G_0/G_1 phase, S phase and G-2M phase. Proliferation index (PI) of the cell was calculated as: $PI=(S+G_2M)/(G_0/G_1+S+G_2M)\times100\%$.

MTT colorimetric analysis[2] Suspend the large intestine carcinoma SW480 cells in logarithmic

growth stage in 100g/L bovine serum culture solution to a concentration of 5×10^8/L. The suspension was inocula ted in to a 96-well plate (100 μL/well) and cultivated for 24 h. After the supernatant was wiped off, 100 μL of 5 g/L bovine serum culture solution was added to each well and the first well as zero control, the second one as cell control, which were added with 100 μL of 5 g/L bovine serum culture solution respectively. From the third one on, 100 μL different drugs with different concentrati on were added to each well as follows and repeated 8 times: PG group: the concentration was 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L, respectively. PG (25 mg/L)+PGL group: the concentration of PGL was 8.00, 16.00, 32.00, 64.00 and 128.00 mg/L. They were cultivated in 37 ℃, 50 g/L CO_2 incubator for 72h, each well added with 10 μL of 5 g/L MTT solution 6 hours before termina ting the culture and 100 μL of 200 g/LSDS solution was added at the termination, kept overnight in the incubator, and the absorptivity (A value) at the 570 nm wavelength was determined on the instrument the next day.

Determination of IP_3 content in carcinoma cell Add 15 mCi/L of [^{3}H]-inosito into the suspension of SW480 cell line, cultivate them in 37 ℃, 50 g/L CO_2 incubator for 18 hours, then add LiCl solution, 2 hours later, add 100μL different drugs with different concentration to each well and cultivate 1 minute as follows: PG group: the concentration was 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L, respectively; PG (25 mg/L)+PGL group: the concentration of PGL is 16.00, 32.00 and 64.00 mg/L. Separate IP_3 from the suspension with anion change separation column according to the method described in reference[3,4], then determine its CPM value with hydroflicker.

Determination of PKC activity Determine the PKC activity in human colonic carcinoma SW480 cell line under the effect of PG and PGL according to the method described in reference[5] with isotopic enzymolysis. The grouping and concentration of PG and PGL were the same with IP_3 determination.

Determination of Ca^{2+} concentration Determine the Ca^{2+} concentration in human colonic carcinoma SW480 cell line under the effect of PG and PGL according to the method described in reference[6] with flu orometry. The grouping and concentration of PG and PGL were the same with IP_3 determination.

Specimen Fourty-eight cases of radical resection of colorectal carcinomas collected from August 1996 to April 1997 in our department were chosen as experimental materials. Ten cases of normal colorectal mucosa served as c ontrol group, including 3 cases of inner prolapse of rectum, 2 of volvulus of sigmoid and 5 of sudden death. All the specimens were confirmed by pathological ex amination. Fresh colorectal carcinoma tissue (3 g) and mucosas 3 cm and 6 cm adjacent to carcinoma were cut respectively, fixed with 40 g/L pa raformaldehyde, dehydrated and embedded with paraffin.

Immunohistochemistry method Immunohistochemistry staining of gastrin, c-myc, c-fos and rasP21 was carried out by SP method, referring to the illustration of reagent kit. The first antibody dilution of gastrin was 1:50 , while the dilution of c-myc, c-fos and rasP21 was all 1:100. Microwav e was used to repair the protein of c-myc, c-fos and rasP21. Normal mucosa of antrum was taken as positive control for gastrin, positively stained tissue of hepatic carcinoma served as positive control for c-myc, c-fos and rasP21. PBS solution replacing the first antibody was taken as negative control. Cells containing brown-yellow pellets were considered as positive cells.

Argyrophilia nucleolar organizer regions count (AgNORs) Argyr ophilia staining was carried out according to Hu PH's modified method[7] and observed under ×100 oil microscope. The nucleus background appeared light yellow while the AgNORs granule appeared palm-black. The amount of AgNORs granule of 50-200 cells and the mean value were calculated.

Statistics All the data was expressed as mean± standard deviation ($\bar{x}\pm s$), and analysed with *t* test or one-way analysis of variance; the differences between the rates of different groups were analysed by χ^2 test.

RESULTS

Model of transplanted human colonic carcinoma in gymnomouse

The inoculation of transplanted carcinoma was 100% successful, no gymnomouse died. The inoculation period was 6-8 days, the speed of growth became stable till the sixth generation. At the end of the 5th week, the long diameter of the mass reached 1.6 cm-2.0 cm. It was elliptical in shape and smooth on surf ace in the early stage; while in the advanced stage, the shape became irregular and the surface became nodal. The histological *H.E.* stain and ultrastructure of the transplanted carcinoma had the same pathological feature of human colonic carcinoma.

Effect of PG, PGL and SMS on the transplanted

human colonic carcinoma in gymnomouse

All the gymnomice bearing the transplanted carcinoma survived at the end of the experiment. The volume, weight, cAMP, DNA and protein content in carcinoma cell, cell amount and proliferation index of S and G_2M phase in PG group were significantly higher than those of control group (P<0.05-001), markedly lower in PGL and PG +PGL group in PG group (P<0.05-0.01), yet there was no st atistical difference between PGL, PG+PGL groups and control group (P>0.05) ; and markedly lower in SMS and SMS+PG group than in PG group and control group (P<0.01). The cell amount of G_0/G_1 phase in PG group was obviously lower than in control group (P<0.01), markedly higher in PGL and PG+PGL gro up than in PG group (P<0.01), without statistical difference between PGL, PG+PGL groups and control group (P> 0.05); markedly higher in SMS and SMS+P G group than in PG group and control group (P< 0.01, Tables 1-3).

Table 1 Effect of PG, PGL and SMS on the volume and weight of transp lanted carcinoma ($\bar{x}$±s, n = 5)

Group	Volume (mm^3)	Weight (g)
Control	1766±36	3.04±0.13
PG	1926±98[a]	3.37±0.21[a]
PGL	1750±53[d]	2.98±0.16[c]
SMS	210±13[bd]	0.90±0.14[bdaa]
PG+PGL	1708±59[d]	2.91±0.23[c]
PG+SMS	224±19[bd]	0.95±0.12[bd]

[a]P<0.05, [b]P<0.01 *vs* control group; [c]P<0.05, [d]P<0.01 *vs* PG group.

Table 2 Effect of PG, PGL and SMS on cAMP, DNA and protein content ($\bar{x}$±s, n = 5)

Group	CAMP(pmol/mL)	DNA(dalton)	Protein(dalton)
Control	2.74±0.14	947±16	364±12
PG	3.18±0.23[b]	1004±17[b]	675±18[b]
PGL	2.72±0.15[d]	940±21[d]	356±9[d]
SMS	1.87±0.14[bd]	684±13[bd]	272±11[bd]
PG+PGL	2.82±0.17[c]	940±25[d]	369±14[d]
PG+SMS	1.86±0.17[bd]	687±21[bd]	274±13[bd]

[b]P<0.01 *vs* control group; [c]P<0.05, [d]P<0.01 *vs* PG group.

Table 3 Effect of PG, PGL and SMS on cell cycle and proliferation index (PI)

Group	G_0/G_1 (%)	S (%)	G_2M (%)	PI (%)
Control	64.92±1.72	18.24±1.20	16.84±2.35	35.08±1.72
PG	59.22±1.18[b]	20.16±1.06[a]	20.62±2.05[a]	40.78±1.81[b]
PGL	67.18±2.23[d]	18.04±1.43[c]	14.78±1.09[d]	32.82±2.27[d]
SMS	80.04±2.29[bd]	14.90±1.46[bd]	5.06±1.61[bd]	19.96±2.39[bd]
PG+PGL	67.76±2.41[d]	18.10±1.40[c]	14.34±0.66[d]	32.24±2.41[d]
PG+SMS	80.26±2.73[bd]	15.22±1.78[bd]	4.54±1.25[bd]	19.22±2.73[bd]

[a]P < 0.05, [b]P < 0.01 *vs* control group; [c]P < 0.05, [d]P < 0.01 *vs* PG group.

Effect of PG on the amount of viable cells (A value), IP_3 content (CPM) and Ca^{2+} concentration in cell and membrane PKC activity of SW480 cell line

When PG was at the concentration of 12.5 mg/L, the amount of viable cells, IP_3 content and Ca^{2+} concentration in the cells of PG group were markedly higher than those in control group (P< 0.05) while it was at the conce ntration of 25 mg/L, they all reached the highest value, but plasma PKC a ctivity decreased, and all had statistical difference from those of control grou p (P<0.05-0.01). When PG concentration exceeded 50mg/L, these items did not continue to increase and plasma PKC activity did not continue to decrease, but they were all statistically different from those of control group (P<0.05-0.01, Table 4).

Effect of PG+PGL on the amount of viable cells (A value), IP_3 content (CPM) and Ca^{2+} concentration in cell and membrane PKC activity of SW480 cell line

When PGL was at the concentration of 8 mg/L, the amount of viable cells (A value) of PG (25 mg/L)+ PGL group was markedly smaller than that of PG group (P<0.01), when at the concentration of 16 mg/L, the amount of viable cells, IP_3 content and Ca^{2+} concentration in cell and membrane PKC activity of PG+PGL group decreased and plasma PKC activity increased, all being statistically different from those of control group (P<0.05-0.01). At the concentration of 32mL/L, they decreased to the lowest value and were markedly lower than those in PG group (P < 0.05-0.01), and did not differ significantly from those of the control group (P>0.05). At the concentration of 64mg/L, these it ems in PG+PGL group did not continue to decrease, but they all had statistical difference from those of PG group (P<0.05 - 0.01, Table 5).

Expression of gastric, c-myc, c-fos and rasP21 and AgNORs count in carcinoma tissue

The positive expression rate of gastrin, c-myc, c-fos and rasP21 in 48 cases o f carcinoma tissue was 39.6%, 54.2%, 479% and 54.2% respectively and signif icantly higher than that in mucosa 3cm and 6cm adjacent to carcinoma tissue and normal colorectal mucosa (P < 0.01). The positive expression rate of gastrin of highly-differentiated adenocarcinoma was significantly higher than that of poorly-differentiated and mucinous adenocarcinoma (P<0.05), there was no statistical difference in the positive expression rate of c-myc, c-fos and rasP21 between groups of different pathological types (P>0.05). The AgNORs count of carcinoma tissue was significantly higher than that in mucosa 3 cm and 6 cm adjacent to carcinoma tissue and normal colorectal mucosa (P < 0.01); and the count of mucosa 3 cm adjacent to carcinoma

tissue was signi ficantly higher than that in mucosa 6cm adjacent to carcinoma tissue and normal colorectal mucosa (P<0.05, Tables 6-7).

Relationship between gastrin and the expression of c-myc, c-fos, rasP21 and AgNORs count in carcinoma tissue

The positive expression rate of c-myc and c-fos and the AgNORs count in gastrin-positive group was significantly higher than those in gastrin-negative group ($P < 0.05 - 0.01$), while the positive expression rate of rasP21 in gastrin -positive group was not different from that in gastrin-negative group (P >0.05, Table 8).

Table 4 Effect of PG on VCC, IP_3, $[Ca^{2+}]$ and PKC activity (pmol/min per mg protein) ($\bar{x}$±s, n = 5)

Group(mg/L)	VCC(*A*)	IP_3(CPM)	$[Ca^{2+}]$I(nM)	Plasma PKC	Membrane PKC
Control	1.554±0.009	8.83±1.33	26.36±2.91	2.39±1.30	1.07±0.28
PG6.25	1.555±0.005	10.16±1.33	30.16±2.16	2.36±0.84	1.09±0.16
12.50	1.563±0.010[a]	11.18±0.75[a]	50.69±2.30[a]	2.30 ±0.45	1.15±0.15
25.00	1.580±0.011[b]	16.17±0.75[b]	101.44±2.49[b]	0.91±0.34[a]	2.65±1.21[a]
50.00	1.579±0.008[b]	14.10±1.60[b]	70.63±4.17[b]	0.92±0.26[a]	2.65±0.60[a]
100.00	1.578±0.010[b]	13.00±2.53[b]	62.59±2.59[b]	0.91±0.14[a]	2.66±0.68[a]

[a]P<0.05, [b]P<0.01 *vs* control group.

Table 5 Effect of PG+PGL on VCC, IP_3,$[Ca^{2+}]$i and PKC acti-vity (pmol/min per mg protein) ($\bar{x}$±s, n = 5)

Group	PG(mg/L)	PGL(mg/L)	VCC(*A*)	IP_3(CPM)	$[Ca^{2+}]$i(nM)	Plasma PKC	Membrane PKC
Control	0.00	0.00	1.554±0.009	8.83±1.33	26.36±2.91	2.39±1.30	1.07±0.28
PG	25.00	0.00	1.580±0.011[b]	16.17±0.75[b]	101.44±2.49[b]	0.91±0.34[a]	2.65±0.21[a]
PG+PGL	25.00	8.00	1.553±0.016[d]				
	25.00	16.00	1.551±0.008[d]	9.17±1.47[d]	32.63±2.86[d]	2.33±0.29[c]	1.05±0.09[c]
	25.00	32.00	1.546±0.011[d]	9.00±1.58[d]	31.79±4.41[d]	2.30±0.61[c]	1.05±0.20[c]
	25.00	64.00	1.549±0.011[d]	9.33±1.97[d]	32.45±2.46[d]	2.32±0.17[c]	1.09±0.12[c]
	25.00	128.00	1.549±0.014[d]				

[a]P<0.05, [b]P<0.01 *vs* control group; [c]P<0.05, [d]P<0.01 *vs* PG group.

Table 6 Expression of gastrin, c-myc, c-fos, rasp21 and AgNORs

Group	*n*	gastrin positive (%)	c-myc positive (%)	c-fos positive (%)	rasp21 positive (%)	AgNORs
Carcinoma tissue	48	19(39.6)[b]	26(54.2)[b]	23(47.9)[b]	26(54.2)[b]	7.10±1.48[b]
3cm mucosa	48	2(4.2)	12(25.0)	9(18.8)	10(20.8)	3.65±1.04[c]
6cm mucosa	48	0(0)	7(14.6)	4(803)	6(12.5)	2.88±0.73
Normal	10	0(0)	1(10.0)	1(10.0)	2(20.0)	2.85±0.60

[b]P<0.01 *vs* 3cm, 6cm and normal mucosa group. [c]P<0.05 *vs* 6cm and normal mucosa group.

Table 7 Expression of gastrin, c-myc, c-fos, rasp21 and AgNORs in different pathological carcinoma tissue

Group	*n*	Gastrin positive (%)	c-myc positive (%)	c-fos positive (%)	rasp21 positive (%)	AgNORs
Highly-differentiated	26	14(53.9)[a]	15(57.7)	13(50.0)	15(57.7)	7.12±1.80
Moderately-differentiated	12	4(33.3)	5(41.7)	7(58.3)	6(50.0)	7.25±0.86
Poorly- and mucinous	10	1(10.0)	6(60.0)	3(30.0)	5(50.0)	7.11±1.20

[a]P<0.05 *vs* poorly-differentiated and mucinous group.

Table 8 Relation between gastrin and c-myc, c-fos, rasp21 and AgNO Rs

Group	*n*	c-myc positive(%)	c-fos positive(%)	rasp21 positive(%)	AgNORs
Gastrin-positive	19	15(78.9)[a]	14(73.7)[b]	11(57.9)	7.84±1.30[b]
Gastrin-negative	29	11(37.9)	9(31.0)	15(51.7)	6.22±1.40

[a]P<0.05, [b]P<0.01 *vs* gastrin-negative group.

DISCUSSION

The main physiological function of gastrin is to stimulate the secretion of gastric acid and nourish the gastrointestinal mucosa. In recent years, it has been found that some gastrointestinal carcinomas could express gastrin gene[8,9] and there existed gastrin receptor on the carcinoma cell membrane, and gastrin could stimulate the growth of gastrin carcinoma. So, more and more researchers focus their interest on the relationship between gastrin and colonic carcinoma.

The transplanted human colonic carcinoma model from SW480 cell line in gymnomouse established by us remained the biological characteristics of colonic carcinoma. After subcutaneous injection of 8μg pentagastrin per day for 35 days, the volume and weight of transplanted carcinoma, the cAMP content in carcinoma cell were significantly higher than those of control group, indicating that gastrin promoted the proliferation of transplanted colonic carcinoma. Ishizuka[10] reported that the combination of gastrin and receptor could increase the content of cAMP in carcinoma cell, then transduct external information into cell through the cAMP or protein kinase C pathway to regulate the cell growth and differentiation. Mauss[11]found that the promoting effect of gastrin on colonic carcinoma cell was selective, margarapeptide gastrin stimulated the growth of HT29, LoVo, CoLo32 cell line, but inhibited the growth of T84, HCT116 cell lines. The difference depended on the quality and quantity of cAMP-dependent protein kinase. The increased expression of Type I cAMP protein kinase could promote the differentiation and growth of carcinoma cells, while the decreased expression of Type IcAMP protein kinase or the increased expression of Type II cAMP protein kinase could inhibit the differentiation and growth of carcinoma cells[12]. Baldwin[13] reported that gastrin could promote the growth of 50 percent of the transplanted colonic carcinoma *in vivo*. We also determined the DNA, protein content and cell cycle by flow-cytometry, and found that DNA and protein content in carcinoma cell and the cell amount and proliferation index of S and G_2M phase of PG group were significantly higher than those of control group, while the cell amount of G_0/G_1 phase of PG group was significantly lower than that of control group. It indicated that the mechanism for the promoting effect of gastrin on the cell division and proliferation of the human colonic carcinoma SW480 cell line might be that gastrin could promote the synthesis of cAMP, DNA and protein in carcinoma cells, the cell growth from G_0/G_1 phase to S and G_2M phase, and regulate the cell cycle of colonic carcinoma cells after receptor.

Venter[14] firstly found that there existed high appetency gastrin receptor on the membrane of human colonic carcinoma cell line LoVo and MC-26, both the nutritious effect of gastrin on the mucosa of normal large intestine and the growth-promoting effect of gastrin on large intestine carcinoma were realized through the combination of gastrin and its receptor on the membrane. We found by MTT colorimetric analysis that pentagastrin could promote the increase of viable cell count of colonic carcinoma SW480 cell line and the effect had a dose-effect dependent relationship with the concentration to some extent, along with the in crease of dosage of pentagastrin, its growth-promoting effect did not continue to increase, but inclined to a stable level. This f inding was consistent with the receptor theory, i.e., the receptor had saturation and indirectly proved that the growth promoting effect of gastrin on large intestine carcinoma was intermediated by gastrin receptor.

Some researches indicate that gastrin is a kind of autocrine growth-promoting factor. Hoosein *et al*[15] found that after the colonic carcinoma HCT1 16 and CBS cell line were cultured for 72 hours, the concentration of gastrin was 10.15pg/10^6 cell. Finley *et al*[16] found by the immunocytochemi cal method that at least 50% of the colorectal carcinoma cells expressed gastrin. In our experiment, 19 of 48 cases of colorectal carcinoma expressed gastrin, with a positive rate of 39.6%, which was significantly higher than that in mucosa adjacent to carcinoma and normal mucosa, the expression rate of gastrin in highly-differentiated group was the highest (53.9%), all these indicated that so me colorectal carcinomas could produce gastrin in an autocrine manner. Translational processing of gastrin mRNA to precursor forms of gastrin needs the participation of multiple enzymes and cofactors to produce mature gastrin finally. Therefore, the sound processing enzymes such as peptidylglycine α-amidating monooxygenase and cofactors in well differentiated carcinoma cells may contrib ute to the production of mature gastrin, while the lack of other cofactors or en zymes in poorly-differentiated carcinoma cells may contribute to incomplete processing of precursor forms of gastrin and the difficiency of mature gastrin[17]. So, the gastrin expression of highly-differentiated adenocarcinoma was obviously higher than that of poorly-differentiated one.

We firstly applied the AgNORs technique to the clinical study of the growth-promoting effect of gastrin on large intestine carcinoma. The AgNORs count could mirror the structure and function of nucleolus, the transcription activity of rRNA and the cell proliferation. The AgNORs count of

colorectal carcinoma in our study was significantly higher than that of mucosa adjacent to carcinoma and normal mucosa, while the count of mucosa 3cm adjacent to carcinoma was higher than that of mucosa 6cm adjacent to carcinoma and normal mucosa. It indicated that the DNA in carcinoma cell and mucosa 3cm adjacent to carcinoma was in a disorder state, the regulation of cell proliferation was uncontrolable. We also found that the AgNORs count of gastrin-positive group was significantly higher than that in gastrin-negative group, indicating that endogenous gastrin had growth-promoting effect on some kinds of colorectal carcinomas.

Oncogene c-myc and c-fos is a kind of effect protein of the karyomitosis signal, which can trigger and regulate the transcription of the genes related with proliferation, besides, c-fos can also regulate its own gene expression with a positive feedback and promote the mitosis and proliferation of the cells[18]. Oncogene ras, a kind of GPT protein located at cell plasma and membrane, participates in the signal transduction regulation of various growth factor recept ors. Once being mutant, ras oncogene will be continously activated and obviously promote the mitosis of the cells[19]. Our results revealed that the positive expression rate of gastrin, c-myc, c-fos and rasP21 in carcinoma tissue was significantly higher than that in mucosa adjacent to carcinoma tissue and normal colorectal mucosa, the positive expression rate of c-myc and c-fos in gastrin-positive group was significantly higher than that in gastrin-negative group. It indicated that the growth-promoting effect of gastrin on colorectal carcinoma may be correlated with the activation of oncogenes. Wang *et al*[20] found that administration of gastrin resulted in the rapid appearance of c-myc mRNA in IEC-6 cells, the maximum increase in c-myc mRNA levels was 7.5-fold that of the normal value. Andrea *et al*[21,22]reported that gastrin had a promoting effect on the growth of AR4-2J cell line, and could induce the increase of c-fos mRNA content; after having combined with its receptor, gastrin trige red a series of phophorylation in PKC signal pathway and induced the activication of extracellular signal regulatory kinase ERK 2, the kinase increased the tra nscription activity of EIK-1, then enlarged the expression of c-fos gene and stimulated the cell proliferation. Seva *et al*[23] reported that gastrin stimulated MAP kinase activation in a dose- and time dependent manner, rasP21 may link the MAP kinase pathway to gastrin receptors to trigger the activation.

Phosphatidylinositol signal pathway played an important role in the biological transmembrane transduction, which was intimately correlated with cell proliferation and tumorgenesis[24]. Phosphatidylinositol-4, 5-bisphosphate (PIP-2) was the direct precursor of second messengers IP_3 and PG, and it functioned through IP_3-Ca^{2+} pathway and DG-PKC pathway. The ascendance of dissociate Ca^{2+} concentration was an important mitosis promoting signal and Ca^{2+} correlated with the genesis and growth of many kinds of carcinomas. Protein kinase C was a kind of important kinase in the phosphatidylinositol signal path way, it existed in plasma of static cells by a non activation form, and once it was activated, it moved to cell membrane. The changes of activity were intimately correlated with cell proliferation. In our study, after we applied pentagastrin to colonic carcinoma SW480 cell line, the VCC, intracellular IP_3 content and Ca^{2+} concentration and membrane PKC activity all ascended and were dose-d ependant obviously. But when pentagastrin and antagonist of gastrin were taken together, they remained changed, demonstrating that the growth-promoting effect of pentagastrin on human colonic carcinoma SW480 cell line was correlated with phosphatidylinositol signal pathway and it was through its receptor intermediate function in phosphatidylinositol signal pathway that proglumide antagonized the growth-promoting effect of gastrin on carcinoma cell. Ishizuka *et al*[10] suggested that the combination of gastrin and its receptor activated the membrane phospholipase and then hydrolyzed phosphatide to PI and DG, the latter then activated PKC, and finally Ca^{2+} was released and functioned.

All the findings above and our results indicated that some carcinoma cells produced gastrin by autocrine, after being intermediated by the second messenger in the cells, the expression of oncogene c-myc and c-fos was enlarged, thus promoting the proliferation of the carcinoma cells.

To block any link in the function procedure of gastrin could weaken or inhibit the growth-promoting effect of gastrin on large intestine carcinoma. There were three kinds of gastrin receptor antagonists: proglumide, somatostatin and prostaglandin (PG). Most of the interests were focused on proglumide, amide, the functional group of proglumide could particularly compete gastrin receptor with gastrin. In our research, 10mg proglumide was injected subcutaneously into transplanted carcinoma in gymnomice, 35 days later, compared with control group, there was no statistical difference in the volume, weight, intracellular cAM P, DNA, protein content and cell cycle of transplanted carcinoma in the PG group, but all the value above including cell amount of S and G 2M phase, proliferat ion index of PG and proglumide group were significantly lower than those of PG group, while the cell amount of G_0/G_1 phase was higher than that of PG group. All these

showed that proglumide had no effect on the growth of human colonic carcinoma SW480 cell line but could inhibit the growth-promoting effect of gastrin on transplanted carcinoma. Proglumide has been applied to the clinical practice. K ameyama *et al*[25] reported that seven large intestine carcinoma patients with hepatic metastasis were treated with proglumide (three times daily, each time 400mg) and 5'DFUR (800mg) for 2 years after the resection of the hepatic metastatic mass, at the same time, patients received chemotherapy by hepatic artery encheiresis and followed up for an average of 39 months. The relapse rate of the proglumide+ chemotherapy group was 14% (1/7), while the rate of the chemot herapy alone group was 52% (14/ 26), so they drew a conclusion that the hormono therapy could effectively prevent the relapse after the resection of the hepatic metastatic mass. We used proglumide as an adjuvant drug to treat 25 colorectal patients having radical resection, besides administration of 400mg proglumide three times a day, MFA chemotherapy program was undertaken at the same time, while 25 patients as control group undertook MFA chemotherapy program alone. Follow-up results indicated that the 3-year survival rate of proglumide group (80%) was higher than that in control group (64%), the relapse or metastasis rate of proglumide group (12%) was lower than that in control group (20%), but without statistical differences ($P>0.05$). The 3-year survival rate of Duke's C and D patients in proglumide group (73.3%) was obviously higher than that in control group (42.8%) ($P<0.05$). It indicated that administration of proglumide as adjuv ant therapy for patients with colorectal carcinomas, particularly the middle and late stage ones, could decrease the relapse or metastasis rate and prolong the survival period. But being a kind of weak gastrin receptor antagonist, only when the gastrin receptor level was high, could proglumide have an inhibitory effect. Therefore, its curable effect on large intestinal carcinoma awaits further evaluation more clinical observations.

Somatostatin is a kind of annular peptide hormone secreting by D cell. The main effect of somatostatin is to inhibit the growth, secretion and absorption of the mucosa of the gastrointestinal tract and to inhibit the release of gastrin, secretin, glucagon and growth hormone. Itzeu *et al*[26] found with immunohistochemistry that there existed D cell in the mucosa of colorectal carcinoma, and suggested that D cell in mucosa of large intestine might have local regulatory effect on the secretion of other hormones. Dy *et al*[27] reported that SMS 20.995 could significantly and concentration-dependently inhibit the growth of transplanted human colonic carcinoma from LIM2405 and LIM2412 cell line. We found that the volume, weight, DNA and protein content in carcinoma cell, cell amount and proliferation index of S and G_2M phase in SMS group and SMS+PG group were markedly lower than those in PG group and control group, and markedly higher in PG group than those in control group. The cell amount of G_0/G_1 phase in SMS group and SMS+PG group was significantly higher than that in PG group and control group. This demonstrated that octapeptide somatostatin had a negative regulatory effect on the transplanted carcinoma, and it could not only inhibit the growth of transplanted human colonic carcinoma from SW480 cell line directly but also inhibit the growth-promoting effect of gastrin on the transplanted carcinoma. The half-life period of somatostatin is shorter than two minutes, so it can not be used pharmaceutically, but its analog manually synthesized such as SMS 201-995 and RC-160 have been used in clinical practice. Having treated 55 pati ents with advanced digestive tract carcinomas who could not endure chemotherapy with SMS201-995, Cascinu *et al*[28]found that SMS201-995 could relieve symptom and improve the quality of life and prolong the survival.

Colorectal carcinoma is a common kind of malignant carcinoma. The carcinoma was mostly in middle or advanced stage when patients first came to see a doctor. Treatment of gastrin-sensitive patients with colonic carcinoma with gastrin recept or antagonists such as proglumide and somatostatin as adjuvant therapy is expected to prolong the survival of the patients and to raise the curative effect and to create new approaches for non-cytotoxic therapy such as hormonotherapy of patients with colorectal carcinoma.

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Effect of retinoic acid on the changes of nuclear matrix in termediate filament system in gastric carcinoma cells

LI Qi-Fu

Subject headings stomach neoplasms; tumor cell, cultured; retinoic acid; nuclear matrix-intermediate filment system

Abstract

AIM To explore the relationship between the configuration chan ges of the nuclear matrix-intermediate filament system in cancer cell induced by retinoic acid and the malignant phenotypic reversion of cancer cells.

METHODS The human gastric adenocarcinoma cell line MGc80-3 cells were induced with 10^{-6} mol/L retinoic acid and subcultured at cover slip strip and gold grids. The cells were treated by selective extraction method and prepared for whole mount electron microscopy observation. The samples were examined respectively with scanning and transmission electron microscope.

RESULTS The nuclear matrix filaments and intermediate filament s in MGc80-3 cells were relatively few and scattered, not well-distributed and arranged irregularly. The nuclear lamina was ununiformly thick and compact, connected to the nuclear matrix filaments and intermediate filaments relaxedly. However, the two kinds of filaments were abundant and well-distributed, different in slender and thick form and interweaved into a regular network in the cells induced by 10^{-6} mol/L RA. The nuclear matrix filaments and intermediate filaments were connected closely by the thin and compact fiber-like lamina, and interlaced into a regular network throughout the whole cell region.

CONCLUSION The NM-IF system in MGc80-3 cells had undergone a restorational change similar to those of normal cells after RA inducement. This alternation is an important morphological and functional expression to the malignant phenotypic reversion of cancer cells.

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INTRODUCTION

The nuclear matrix is a fine network in the eukaryotic nucleus and plays an important role in maintaining nuclear morphology, DNA organization, DNA replication and gene expression. The morphological and functional alternations of the nuclear matrix have an important effect on cell proliferation and differentiation[1,2]. It has been shown in previous studies that there were dramatic differences in the configuration and protein composition of the nuclear matrix between cancer and normal cells[3,4]. Moreover, it has been demonstrated that some carcinogens or anti-cancer agents perform their function by affecting the nuclear matrix[5]. These results suggest that abnormal nuclear matrix is closely relevant to cell canceration. However, the alternations of nuclear matr ix during the differentiation induced by cancer cells and its relation to the ma lignant phenotypic reversion of cancer cells are still poorly understood. To explore the correlation between the configurational changes of the nuclear matrix and the malignant phenotypic reversion of cancer cells, a study was made on the alternations of the nuclear matrix-intermediate filament (NM-IF) system in human gastric adenocarcinoma cell line MGc80-3 induced by retinoic acid (RA).

MATERIALS AND METHODS

Cell culture and induced treatment

MGc80-3 cells were cultured in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum, and an appropriate amount of penicillin, streptomycin and kanamycin. The MGc80-3 cells were induced by the medium containing 10^{-6}mol/L all-*trans*-retinoic acid (RA) (purchased from Sigma Chemical Co.). Then, MGc80-3 cells and the cells treated with RA were seeded in small penicillin bottles with cover slip strip on which some gold grids covered with formvar and coated with carbon were sticked with polylysine, and grown in the

normal medium or the medium containing 10^{-6} mol/L RA respectively. Cells were incubated at 37 ℃ in 5% CO_2 atmosphere for 72 hours.

Cell selective extraction

The cells were selectively extracted as described by Capco[6]. MGc80-3 cells and the cells treated with RA were rinsed with D-Hank's solution twice at 37 ℃, and extracted by high ionic strength extraction solution (10 mmol/L PIPES, pH 6.8, 250 mmol/L $(NH_4)_2SO_4$, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 1.2 mmol/L PMSF, 0.5% Triton X-100) at 4 ℃ for 3 min. The extracted cells were rinsed in non-enzyme digestion solution (10 mmol/L PIPES, pH 6.8, 50 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 1.2 mmol/L PMSF, 0.5% Triton X-100), and digested in digestion solution containing DNase I (400mg/L) and RNase A (400 mg/L) for 20 min at 23 ℃. The samples were placed in high ionic strength extraction solution at 23 ℃ for 5 min. So far only the nuclear matrix- intermediate filament structure remained intact.

Sample preparation for the whole mount electron microscopy

The NM-IF samples produced by selective extraction were prefixed in 2% glutaraldehyde (made in non-enzyme digestion solution) at 4 ℃ for 30 min. The samples were then rinsed with 0.1 mol/L sodium cacodylate buffer (pH 7.4), postfixed in 1% O_SO_4 (made in 0.1 mol/L sodium cacodylate) at 4 ℃ for 5 min, dehydrated in ethanol series, replaced in isoamyl acetate, dried through the CO_2 critical point. The cell samples attached to grids were examined with a JEM-100CX II transmission electron microscope (TEM), and the cell samples grown on cover slip strip were gilded in vacuum and examined with a HITACHI S-520 scanning electron microscope (SEM).

RESULTS

It was revealed by TEM and SEM that MGc80-3 cells and the cells induced by RA after selective extraction remained a filament network spreading all over the original cell region and structurally interlinked. In addition, the original nucleus region was maintained by the nuclear lamina and formed an interlinking and integrated NM-IF system (Figures 1-3). After induced treatment with RA, the changes of NM-IF system in MGc80-3 cells were observed.

Nuclear matrix

The nuclear matrix filaments in MGc80-3 cells were relatively few and scattered, not well-distributed and arranged irregularly within the nucleus region. The nuclear matrix filaments were short, and there were few single filaments while most of the nuclear matrix filaments were quite thick in bundle-like form and interweaved into an irregular network or in a flocculent structure. One or more residual nucleoli were usually observed within the nucleus region and maintained by a few of nuclear matrix filament bundles (Figures 1, 2). However, in the MGc80 -3 cells induced by RA, the nuclear matrix filaments were abundant and well-distributed, different in slender and thick form in the nucleus region. The nuclear matrix filaments were slender, in which the single filaments increased, and interweaved into a regular network. The nuclear matrix filaments or filament bundle s were arranged radiately and connected to the residual nucleoli (Figures 3-5).

Nuclear lamina

The nuclear lamina in MGc80-3 cells was ununiformly thick and compact. The inner nuclear lamina was nonected with some thick nuclear matrix filament bundles or thin and short filaments. It could always be seen that the intermediate filament bundle and the thin or thick filament of its branches were connected to and terminated on the outer nuclear lamina. Nevertheless, the nuclear matrix filaments and the intermediate filaments connected to the nuclear lamina were relatively few and scattered (Figures 1, 2). The nuclear lamina in MGc80-3 cells induced by RA turned into a thin and compact fibroid structure. The inner nuclear lamina was connected closely with the nuclear matrix filaments, and many long and slender intermediate filament bundles were terminated directly on the outer nuclear lamina. Both the nuclear matrix filaments and the intermediate filaments connected to the nuclear lamina increased and appeared quite densely. It impelled the three parts to link up with each other more closely (Figures 3, 5).

Intermediate filament

The amount of intermediate filaments in MGc80-3 cells was rather small. They were present mainly in the cytoplasm region around the nucleus and only a few in t he peripheral region within cytomembrane, and were not well-distributed. The intermediate filaments in which single filaments were few, were chiefly in thick bundles or in strip-rope-like structure, and arranged irregularly (Figures 1, 2). But they were abundant and well-distributed in the cytoplasm in MGc80-3 cell s induced by RA. They spread from the region around nucleus and to the cellular edge. Quite a few single filaments were found in intermediate filaments which in terweaved with the slender intermediate filament bundles into a well-distributed and regular network throughout the cytoplasm region (Figures 3, 5, 6).

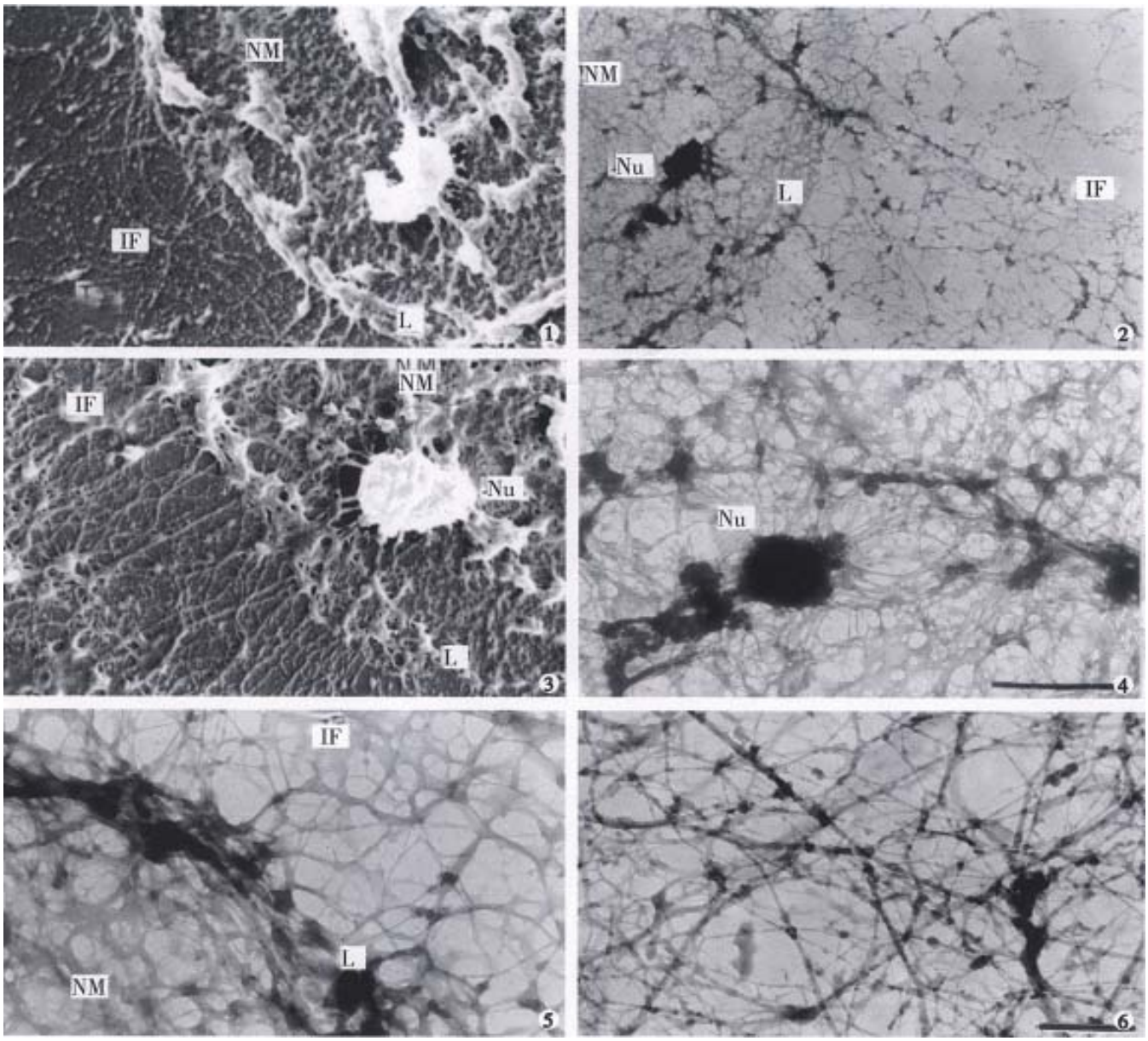

Figure 1 SEM observation of NM-IF system in MGc80- 3 cells (L lamina). Bar=1μm
Figure 2 TEM observation of NM-IF system in MGc80-3 cells (Nu residual nucleolus). Bar=1μm
Figure 3 SEM observation of NM-IF system in RA-treated cel ls. Bar=1μm
Figure 4 TEM observation of NM filament network in RA-treat ed cells. Bar=1μm
Figure 5 NM filament connects closely with intermediate fila ment by thin and regular lamina in RA-treated cells. Bar=0.5μm
Figure 6 The network of plentiful intermediate filaments in RA-treated cells. Bar=0.5μm

DISCUSSION

The abnormality of nuclear matrix is largely associated with the canceration of cell. Previous studies demonstrated that the nuclear matrix in cancer cells has undergone an irregular and abnormal configuration distinguished from that of normal cells[1,3], indicating that the nuclear matrix in cancer cells has some distinctive configuration characteristics which differed significantly from those of normal cells. The observation in this study displayed that the nuclear matrix filaments and intermediate filaments in MGc80-3 cells were relatively few, not well distributed, arranged irregularly, and the single filaments were few while filament bundles were plentiful. The thick nuclear lamina was not largely associated with the nuclear matrix filaments and intermediate filaments. It showed the typical configuration characteristics of nuclear matrix in malignant tumor cells. The NM-IF system in MGc80-3 cells induced with RA had undergone a significant change. The nuclear matrix filaments and intermediate filaments were abundant, well-distributed with the single filaments increased and differed in slender and thick form and

inteweaved into a regular network. Meanwhile, the nuclear matrix and intermediate filaments were connected closely by the thin and compact fiber-like nuclear lamina and organized into an integrated network throughout the cell. The characteristics of this organized and integrated configuration of NM-IF system were significantly different from those of MGc80-3 cells but similar to those of normal cells of epithelial origin[6-7]. It demonstrated that RA could impel the NM-IF system in MGc80-3 cells to exert a reversional configuration alteration. In this regard, the restoration of the normal configuration of nuclear matrix is obviously an important morphological and functional expression to the malignant phenotypic reversion of gastric carcinoma cells.

The nuclear matrix plays a role not only in maintaining nuclear morphology as a framework within the nucleus, but in DNA replication and chromosomal construction. Consequently, the nuclear matrix can directly affect the cell division and proliferation[1,2,8]. It is obvious that the changes of nuclear morphology and the inhibition of DNA synthesis and cell proliferation in MGc80-3 cells induced by RA are associated closely with the configuration and functional alternation of the nuclear matrix. It can be concluded that these effects are resulted from the restoration of normal configuration of nuclear matrix in MGc80-3 cells induced by RA. In addition, the nuclear matrix plays an important influence upon regulating gene expression by acting on gene transcription, RNA processing and modifying, and directional transport[2,9]. Previous studies suggested that oncogene of cancer cells mainly existed in DNA sequences connecting to the nuclear matrix, and the transcription of oncogene couldn't be underway until the oncogene was connected to the nuclear matrix[3,10]. It indicates that the nuclear matrix plays an important role in oncogene expression. Therefore, the restoration of normal configuration and function of nuclear matrix in gastric carcinoma cells induced with RA must have an important effect on regulating the expression of oncogene and tumor suppressor gene which are related to gastric carcinoma cells. For this reason, how to investigate the functional alternations of nuclear matrix in cancer cells during the induced differentiation will have a momentous significance in revealing the mechanism of cell canceration and reversion.

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Gastroesophageal reflux: the features in elderly patients

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Subject headings gastroesophageal reflux; esophagitis; hiatal hernia

Abstract

AIM To compare the features of gastroesophageal reflux disease between elderly and younger patients.

METHODS Twenty-four hour pH-monitoring and endoscopy were per formed for the 66 elderly patients with typical gastroesophageal reflux symptoms, and the results were compared with 112 symptomatic younger patients.

RESULTS The results of 24-h pH-monitoring and endoscopy showe d that the elderly patients had pathological reflux and reflux esophagitis more frequently than the younger patients. Percentage time with pH<4 in elderly patients with reflux esophagitis was 32.5% in 24 hours, as compared with 12.9% in the younger patients with reflux esophagitis ($P<0.05$). The elderly patient s with reflux esophagitis have longer periods of acid reflux in both upright and supine positions than the younger patients. Endoscopy showed that 20.8% of elderly patients had grade III/IV esophagitis, whereas only 3.4% of younger patients had grade III/IV esophagitis ($P < 0.002$). Percentages of grades I/II esophagitis in the two groups were 12.5% and 26.5%, respectively ($P<0.002$).

CONCLUSION Elderly patients, as compared with younger patients, have more severe gastroesophageal reflux and esophageal lesions. The incompetence of lower esophageal sphincter and the presence of hiatal hernia may be important factors leading to the difference in incidence and severity of reflux esophagitis between elderly and younger patients.

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INTRODUCTION

With the introduction of intraesophageal 24-h pH-monitoring in clinical practi ce, it is now possible to identify patterns of gastroesophageal reflux (GER) in the healthy people and patients and to assess the effect of H2 blockers and H^+ /K^+ adenosine triphosphatase (ATPase) inhibitors on GER diseases[1-7]. It is increasingly recognized that symptomatic GER may occur in the patients of all ages. However, little information is available on symptomatic GER patterns in the elderly. Recently, Mold et al, investigated GER disease (GERD) in patients aged over 62 years in a primary care setting[8]. However, the focus of this study was not on patterns of GER, but on prevalence of GER in the elderly. Therefore, the aim of the present study was to identify patterns and features of symptomatic GER in the elderly patients.

PATIENTS AND METHODS

Patients

One hundred and seventy-eight consecutive patients who had experienced heartbur n, regurgitation and chest pain for at least 6 months were studied. These includ ed 66 elderly patients (36 men, 30 women) ranging in age from 65 to 76 years (mean age 67 years), and 112 younger patients (64 men, 48 women) aged from 21 to 64 years (mean age 41 years). None had undergone upper gastrointestinal surgery such as gastric resection and selective proximal vagotomy. None had taken H2-blockers or H^+/K^+-ATPase inhibitors in the 2-week period before 24-h pH-monitoring.

Endoscopy

All patients underwent upper gastrointestinal endoscopy. Esophagitis grade was assessed endoscopically by using the Savary & Miller Criteria[9], that is, from grade I to IV. Of these patients, very few presented with grades II and III esophagitis, and therefore grade I and II, and III and IV were grouped together.

Twenty-four hour intraesophageal pH-monitoring

Twenty-four hour intraesophageal pH-monitoring was carried out by a routine method used in our laboratory[10]. Patients were advised to take a stand ard 2200 kilocalories meal during 24-h

intraesophageal pH-monitoring. A glass pH-electrode with an incorporated potassium chloride reference electrode (Ingol delectrode, No 440) was introduced via the naso-esophageal route and positioned with the tip 5 cm above the gastroesophageal junction identified by the pH-metry[11,12]. The output from the pH probe was recorded on a solid-s tate recorder (Autronicord CM 18), which could be carried on a belt by the patie nts. Data were analyzed on a computer by means of a dedicated computer program. The parameters recorded included the frequency and duration of GER in 24 hours, upright and supine positions, and frequency of GER longer than 5 minutes. Pathological reflux was diagnosed if 1) the pH value in the regurgitated contents was <4.0, and 2) the complete reflux duration was more than 7% in 24 hours[10 -12]. Reflux esophagitis was diagnosed if the patient with pathological GER had inflammatory esophageal lesions.

Statistical methods

Anamnestic data and endoscopic findings were analysed by means of the Chi-square test. The Mann-Whitney test and Fisher exact test were used to evaluate GER parameters.

RESULTS

Endoscopic findings showed that elderly patients had more severe esophageal lesions and a higher incidence of hiatal hernia than younger patients (Table 1) (P<0.002).

On the basis of the results of the endoscopy and intraesophageal 24-h pH-monitoring, patients can be divided into three subgroups: physiologic reflux, pathologic reflux and reflux esophagitis. Table 2 lists the percentages of the three groups in elderly and younger patients. The incidence of pathologic reflux and r eflux esophagitis in elderly patients was higher than that in younger patients (P<0.05).

Figure 1 shows the percentage time of GER episodes in 24 hours. In the reflux esophagitis group, percentage time of GER episodes in the elderly and younger patients during the entire 24-h period was 36.2% and 17.8%, respectively (P <0.05). No statistically significant differences in the percentage time with GER episodes were found between the elderly and younger patients in either the physiologic or pathologic reflux subgroups.

Figures 2 and 3 show the percentage time of GER episodes in upright and supine positions. Elderly patients with reflux esophagitis had significantly greater percentage time of GER episodes than younger patients with reflux esophagitis (upright position, 32.4% versus 13.6% supine position, 30.7% versus 11.7%; P<0.05).

Table 1 Endoscopic findings in elderly and younger patients

	Non-esophagitis (%)	Esophagitis		Hiatal hernia(%)
		I/II(%)	III/IV(%)	
Elderly patients	44(66.7)	8(12.1)[b]	14(21.2)[b]	16(24.2)[b]
Younger patients	78(69.6)	29(25.8)	4(3.6)	17(15.1)

[b]P<0.001, elderly patients *vs* younger patients .

Table 2 Percentages of physiological reflux (PhR), pathological reflux and reflux esophagitis (RE) in elderly and younger patients

	PhR(%)	PR(%)	RE(%)
Elderly patients	24(36.4)[a]	20(30.3)[a]	22(33.3)[a]
Younger patients	59(52.7)	19(17.0)	34(30.3)

[a]P < 0.05, elderly patients *vs* younger patients; PhR = physiological reflux; PR = pathological reflux; RE = reflux esophagitis.

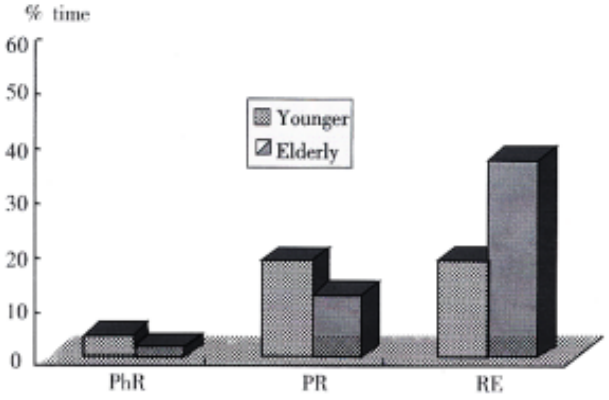

Figure1 Percentige time of GER episodes in 24 hours .
PhR: physiological reflux; PR: pathological reflux; RE: reflux esophagitis.

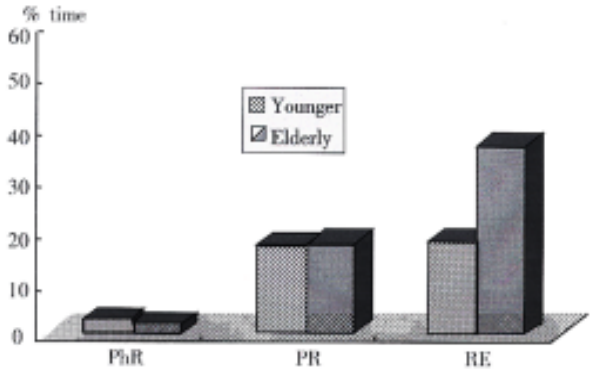

Figure2 Percentage time of GER episodes in upright position.

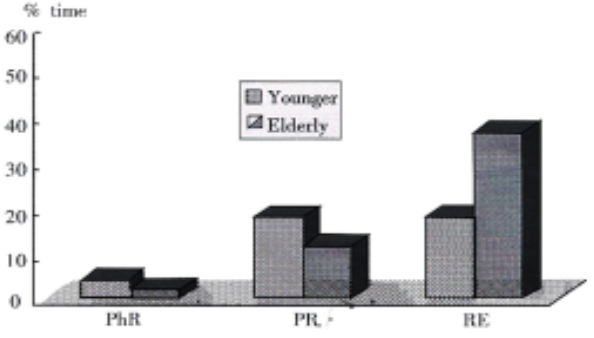

Figure3 Percentage time of GER episodes in supine positions.

Elderly patients with reflux esophagitis had a higher frequency of GER episodes than younger patients with reflux esophagitis, the difference being statistically significant (Table 3). There was no significant difference between elderly and younger patients in frequency of GER episodes lasting more than 5 minutes (Table 4).

Table 3 Frequency of GER episodes in elderly and younger patients ($\bar{x}\pm s$)

	Elderly patients		Younger patients	
Upright position				
PhR	10	10[a]	24	20[a]
PR	45	21[b]	55	21[b]
RE	85	64[c]	53	32[c]
Supine position				
PhR	3	4	3	9
PR	17	9[d]	8	7[d]
RE	18	3[e]	7	7[e]

[a]$P < 0.01$, elderly *vs* younger; [c]$P < 0.05$, elderly *vs* younger; [d]$P<0.01$, elderly *vs* younger; [e]$P < 0.05$, elderly *vs* younger.

Table 4 Frequency of GER episodes lasting more than 5 mintues in eld erly and younger patients ($\bar{x}\pm s$)

	Elderly patients		Younger patients	
Upright position				
PR	3	2	5	3
RE	9	6	5	6
Supine position				
PR	3	2	5	3
RE	3	2	2	2

$P>0.05$, elderly patients *vs* younger patients; PR=pathological reflux; RE=reflux esophagitis.

DISCUSSION

Although several studies of GER patterns have been carried out in healthy subjects and patients, the GER profile in the elderly has not been investigated. Many older people, as a result of physiological change or disease, have decreased salivary flow, gastric acid production, esophageal motility, gastric emptying, and/or lower esophageal sphincter tone. These changes may affect the features of symptomatic GER in the elderly patients.

The present study demonstrated that patterns of GER and esophageal lesions in elderly patients with GER symptoms showed different features from those presented by the younger patients. Firstly, the incidence of pathological reflux and reflux esophagitis in the elderly patients with GER symptoms was significantly higher than in the younger patients (66.7% *vs* 46.9%). Secondly, the elderly patients with GER symptoms have more severe esophageal lesions than younger patients. In the elderly patients, 20.8% of patients had grades III/IV esophagitis, as against only 3.4% of patients in the younger group. In addition, elderly patients had a higher incidence of hiatal hernia than their younger counterparts. Thirdly, 24-h intraesophageal pH-monitoring showed that elderly patients with reflux esophagitis had a more severe acid reflux than younger patients with reflux esophagitis. This is due to prolonged periods of acid reflux in both upright and supine positions. Similarly, in elderly patients with reflux esophagitis, the frequency of GER episodes in both upright and supine positions is higher than that in younger patients with reflux esophagitis. It is generally agreed that esophagitis may be the result of abnormal acid GER in most patients. Our results suggest that more severe patterns of GER in elderly patients leads to more severe esophageal lesions.

The present results showed that there was no statistically significant difference between the elderly and younger patients regarding frequency of GER episodes lasting more than 5 minutes (Table 4). This suggests that an impaired esophageal clearing function is not responsible for the difference in incidence and severity of reflux esophagitis observed between elderly and younger patients. Therefore, different pathogenetic mechanism such as the incompetence of lower esophag eal sphincter and the presence of hiatal hernia may be important factors leading to the difference in incidence and severity of reflux esophagitis between elderly and younger patients.

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Review

Human intestinal and biliary cryptosporidiosis

CHEN Xian-Ming and Nicholas F. LaRusso

Subject headings *Cryptosporidium parvum*; cryptosporidiosis/epidemiology; cryptosporidiosis/immunology; cryptosporidiosis/diagnosis; cryptosporidiosis/therapy

Cryptosporidium parvum (C. parvum) is a coccidian parasite of the phylum *Apicomplexa* that infects the gastrointestinal, biliary and respiratory epithelium of humans and animals[1]. Early reports described a disease in humans characterized by protracted, watery diarrhea occurring in immunosuppressed patients, many with acquired immunodeficiency syndrome (AIDS). Recent epidemiologic studies indicate that cryptosporidiosis may also present as an acute, self-limited diarrheal disease in immunocompetent individuals and may account for 1%-10% of diarrheal disease worldwide[2,3]. Despite the magnitude and severity of cryptosporidial infection, the pathogenesis is poorly understood, and there is currently no effective therapy[3]. In this review, we provide a concise summary of what is known about cryptosporidial infection of the intestinal and biliary tract.

THE PARASITE

Cryptosporidium is a coccidian parasite and one of many genera of the protozoan phylum, Apicomplexa (class Sporozoea, subclass Coccidia). Six species are currently recognized on the basis of differences in host specificity, oocyst morphology and site of infection (*C. parvum, C. muris, C. meleagridis, C. baileyi, C. serpentis* and *C. nasorum*); only C. parvum causes diseases in humans[1,4].

C. parvum has a monoxenous life cycle, all stages of development (asexual and sexual) occurring in one host. The entire life cycle may be completed in as few as 2 days in many hosts, and infections may be short-lived or may persist for months. Once ingested, oocysts excyst in the gastrointestinal tract releasing infective sporozoites. The freed sporozoites attach to epithelial cells and become enclosed within parasitophorous vacuoles, developing attachment organelles (stages referred as trophozoites). The trophozoites then undergo asexual proliferation by merogony and form two types of meronts. Type I meronts form 8 merozoites that are liberated from the parasitophorous vacuole when mature; the merozoites then invade other epithelial cells where they undergo another cycle of type I merogony or develop into type II meronts. Type II meronts form 4 merozoites which do not undergo further merogony but produce sexual reproductive stages (called gamonts). Sexual reproduction occurs by gametogony and both microgamets (male) and macrogametocytes (female) are formed. Macrogametocytes are then fertilized by mature microgamets, and the resultant zygotes undergo further asexual development (sporogony) and form sporulated oocysts containing 4 sporozoites. Most oocysts are thick-walled and are excreted from the host in faecal material; some oocysts, however, are thin walled and have been reported to excyst within the same host leading to a new cycle of development[1]. The presence of these auto-infective oocysts and recycling type I meronts are believed to be the means by which persistent chronic infections may develop in hosts without further exposure to exogenous oocysts[1].

EPIDEMIOLOGY

Infection of *C. parvum* in both immunocompetent and immunocompromised humans occurs worldwide. From prevalence studies, oocyst excretion rates are known to vary between 1%-3% in industrialized countries and 10% in less industrialized nations. Seroprevalence rates are much higher. In developed countries, they vary between 25%-35%, while in the developing world these rates are as high as 60%-90% (Figure 1)[3,5,6]. *C. parvum* infection was reported in 10%-15% of the children with diarrhea in the developing world. It has been reported that 10%-16% of AIDS patients with chronic diarrhea in North America and 30%-50% in the developing world are infected with *C. parvum*[7,8] while the infection mainly occurs in the intestine in both immunocompetent and immunocompromised

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individuals, biliary cryptosporidiosis has only been reported in HIV-infec ted patients, being found in 20%-65% of the patients with so-called AIDS-chola ngiopathy[9].

It has been reported that as low as 10 oocysts of *C. parvum* can cause human diseases. Many instances of human-to-human transmission have been recorded bet ween household and family membranes, sexual partners (both heterosexual and homo sexual), hospital patients and staff, and children attending day care centers. While most cases of transmission involve oocysts derived from faecal samples, contaminated water is a source of infection among international travelers, and outbreaks have been associated with contamination of well water, surface water, swimming pools and public water supplies. *C. parvum* oocysts have been recovered from untreated surface waters, filtered swimming pool water, and most importantly, from treated drinking water. Homosexuals practicing oral-anal and/or anal-genital sex, veterinary personnel and animal handlers are particularly at risk[1]. Immunocompromised individuals with hypogammaglobulinaemia, organ transplanted recipients, patients undergoing bone marrow transplantation and patients on immunosuppressive drugs are also at high-risk[10].

IMMUNOLOGY AND PATHOGENESIS

Immunology

Immunocompetent individuals who become infected generally experience a self-lim ited syndrome and become immune to reinfection. In contrast, severe chronic infe ctions may develop in immunocompromised individuals with either congenital or acquired lymphocyte or γ-globulin deficiencies, suggesting both cell -mediated and humoral immune responses are involved in the resolution of infections and the development of immunity[1].

Immunoserological tests have detected specific IgG, IgM, IgA and even IgE antibodies in acute or convalescent sera from infected patients. Local and secretory antibodies have been detected in association with infections, including IgA antibodies in duodenal fluid[11]. Passively acquired antibodies have also been implicated in the prevention or control of infections. Several epidemiological studies recorded a lower prevalence of infections in breast-fed children than in bottle-fed children. However, the role of serum or secretory antibodies in the resolution of infections is unclear. Neutralizing antibodies against surface membrane determinants of free sporozoites and merozoites reduce infectivity, sug gesting that some antibodies may neutralize intraluminal stages of the parasite. Whether these antibodies would be effective against intracellular developmental stages in which the parasite is covered by host membranes is unknown[1].

Experimental studies using immunocompr-omised SCID mice and nude mice have shown that resolution of a *C. parvum* infection requires B and/or *T lymphocytes*[12]. The identification of cryptosporidiosis as one of the opportunistic infections affecting individuals with AIDS also suggests that immunity to this parasite requires $CD4^+$ T cells. $CD4^+$ T cells of patients with AIDS infected with *C. parvum* show that fulminant and persistent disease only occurs in individuals with $CD4^+$ counts < 50 cells/mm^3. Individuals with $CD4^+$ T counts > 200 cells/mm^3 display only transient disease. Individuals with $CD4^+$ T counts <50 cells/mm^3 have an increased incidence of biliary disease, an indicator of chronic cryptosporidiosis, and a decreased survival when compared to those individuals with higher $CD4^+$ T cell counts[9]. IFNγ is important for resistance to *C. parvum*; the absence of this cytokine in mice with a targeted disruption of the IFNγ gene (gene knockout) results in uncontrolled *C. parvum* infection[13].

Pathogenesis

Histological changes associated with intestinal cryptosporidiosis are relatively non-specific and include blunting of villi, hyperplasia of intestinal crypt cells, and infiltration of inflammatory cells into the luminal propria. Neutrophilic infiltrate, villus blunting, cryptitis, epithelial apoptosis and reactive epi thelial changes in the intestine in AIDS patients with cryptosporidiosis correlate with the intensity of *C. parvum* infection[14]. Biliary cryptospor idiosis is also associated with a non-specific inflammatory response. Histologically, there is a periductal inflammatory response with interstitial edema, mixed inflammatory cell infiltrates, and hyperplasia and dilatation of the periductual glands. The fibrosis that develops around the portal tracts of AIDS patients with chronic cryptosporidial infection can mimic the histologic changes seen in primary sclerosing cholangitis. Autopsy reports and prospective studies have supp orted an etiologic role for the organism in biliary syndromes like sclerosing cholangitis and acalculous cholecystitis[15,16]. However, the pathophysiol ogical mechanisms underlying *C. parvum* infection of intestinal and biliary epithelia are not well understood, and at present our understanding of the pathogenesis is still limited to data from animal experimental studies.

The process by which *C. parvum* sporozoites infect epithelial cells consists of two sequential

steps: ① attachment of sporozoites to the plasma membrane of epithelial cells, a primary event in the initial host-parasite interaction and a prerequisite for the pathophysiological consequences; and ② invasion of sporozoites into host cells by invagination of the host cell plasma membrane, which engulfs and eventually completely surrounds the sporozoite to form a parasitophorous vacuole in which the organism remains intracellular but extracytoplasmic[17-19]. Electron microscopy has confirmed the intracellular but extracytoplasmic location of the organism within a parasitophorous vacuole formed by a continuous covering of microvillous membranes, and nearly all endogenous developmental stages of *C. parvum* are confined to the apical surface of epithelial cells. The parasite contains a unique "attachment" or "feeder" organelle which is prominent at the base of each parasitophorous vacuole. Originally, this feeder organelle was thought to be formed by repeated folding of parasite and host epithelial cell membranes. However, recent electron microscopic studies showed that the dense band of feeder organelle underlying the parasite attachment site represents modified host cell cytoskeleton. This organelle is thought to facilitate the uptake of nutrients by the parasite from the host cell.

C. parvum displays a clear predilection to infect only certain sites within the host. The parasite usually infects epithelia in the intestine, respiratory tract and bile ducts. In the human intestine, the stomach is rarely infected, and the upper small bowel, colon, and rectum are less affected than the mid small bowel[1]. Several cell lines from human or animal intestine are sensitive to *C. parvum*-infection *in vitro*, but the susceptibilities of those cells to infection differ[20]. Sporozoites attach to the cultured host cells by their anterior pole and attachment is dose, time, ion, pH and host cell-cycle dependent, and is inhibitable by antibodies against antigens on the sporozoite surface membrane[21,22]. Recently, we developed an *in vitro* system of biliary cryptosporidiosis, and demonstrated that the infection is both apical plasma membrane and liver cell specific (i.e., C. parvum can infect bile duct cells but not hepatocytes *in vitro*)[19]. These characteristics suggested specific molecules on the surface of both epithelial cells and *C. parvum* sporozoites are involved in the infection process. Previous studies did demonstrate the presence of a galactose-N-acetylgalactosamine (Gal-GalNAc) specific *C. parvum* sporozoite surface lectin which may mediate attachment of sporozoites to host cells[22,23]. Moreover, recent reports have shown that *C. parvum* sporozoite motility depends on the parasite cytoskeleton, and host cell cytoskeleton rearrangement might be involved in the biogenesis of parasitophorous vacuoles[19]. Nevertheless, the molecular mechanisms and specific molecules involved in the initial interaction between *C. parvum* sporozoites and epithelial cells remain obscure. Moreover, essentially nothing is known about how the organism actually invades cells and forms a parasitophorous vacuole, a complex organelle on which the life cycle and possibly the cytotoxicity of the organism is dependent. Based on our observations on the interaction of *C. parvum* with intestinal and biliary epithelial cells *in vitro*, we propose a molecular model for *C. parvum* infection of epithelial cells shown in Figure 2.

When microbes interact with host cells, the result is generally host cell dysfunction. Recent data in a variety of tissues infected with either parasite (such as *Entamoeba histohytica*, *Schistosoma mansoni*, *Trypanosoma cruzi* and *Toxoplasma gondii*), bacteria or viruses are consistent with the concept that microbial pathogens can kill host cells by an apoptotic mechanism. Experimentally, it has been shown that *C. parvum* infection of intestinal and biliary epithelial cell monolayers results in a functional disruption of the monolayers and release of LDH from the cell surface. Epithelial apoptosis and reactive epithelial changes in the intestine in AIDS patients with cryptosporidiosis have recently been shown to be associated with *C. parvum* infection[14]. We found that apoptosis occurs in the epithelial cells adjacent to *C. parvum*-infected biliary epithelia *in vivo* in the gallbladder of a patient with AIDS and biliary cry ptosporidiosis[19]. More recently, we found that *C. parvum* can indu ce apoptosis in the cultured human biliary epithelia[19,24]. These results suggest that *C. parvum*, like some other parasites, is directly cytopathic for epithelia via an apoptotic mechanism, a mechanism which is believed critical in liver diseases like primary biliary cirrhosis, primary sclerosing cholangit is, and hepatitis[25].

Release of cytokines and chemokine plays a critical role in the inflammation associated with microbial infection. Rapid upregulation of the C-X-C chemokine fa mily was found in human intestinal epithelial cells infected with gram-negative or gram-positive bacteria. In an *in vitro* model of intestinal cryptosporid iosis, *C. parvum* induces IL-8 release from infected intestinal epithelial cell monolayers[26]. Release of cytokines and chemokines like IL-8 could be involved in the pathogenesis of inflammation in cryptosporidiosis. However, little is known about the early events following host-parasite interactions that influence the course of cytokine and chemokine upregulation and release.

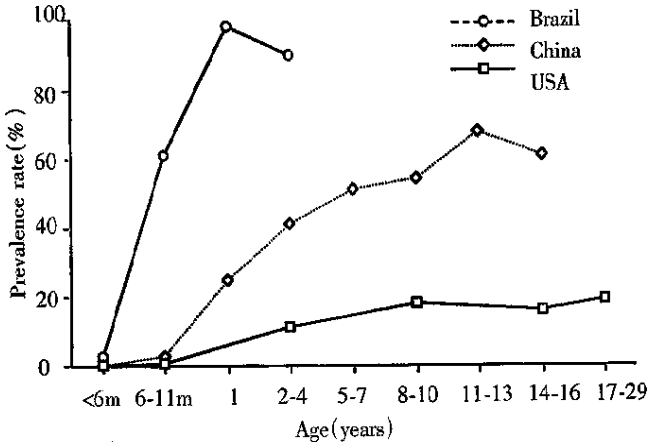

Figure1 Prevalence of IgG antibodies to *C. parvum* in different populations from Brazil, China and the United States. M = month. D ata reproduced from reference 3 with perission.

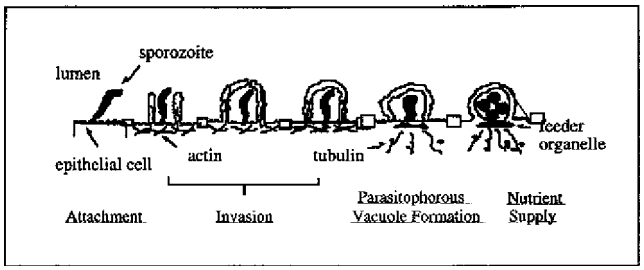

Figure2 Molecular model for *C. parvum* infection of epithelial cells. *C. parvum* sporozoite attaches specifically to the apic al membrane surface of epithelial cells. The attachment induces rearrangement of the host cell cytoskeleton and facilitates the parasite's invasion and parasit ophorous vacuole formation. Host cell cytoskeleton rearrangement forms the feede r organelle underlying the parasite attachment site and facilitates the uptake o f nutrients by the parasite from the host cell cytoplasm.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

Clinical manifestations

The most common clinical manifestation of cryptosporidiosis is diarrhea, characteristically profuse and watery and often containing mucus but rarely blood or leucocytes. Other clinical signs observed include abdominal cramps, low grade fever, nausea and vomiting[10]. The duration and severity of clinical symptoms depend largely on the immune status of the infected individual. In immunocompetent individuals, the disease is usually self-limited. After an incubation period of 7-10 days, >90% of infected cases present with watery diarrhea lasting approximately 2 weeks, 50% present with nausea, vomiting and cramp-like abdominal pain and 36% present with a febrile illness. In immunocompromised individuals, the disease is much more severe. In HIV-infected patients, the disease severity is related to the site of *C. parvum* infection and $CD4^+$ cell count[27]. The diarrhea is watery and stool frequency can be up to 10 times a day with a mean volume of one litter. Individuals with AIDS infected with *C. parvum* can experience a 10% drop in body weight, and usually develop severe malabsorption. Most of them never clear the infection, and ultimately have a shorter survival than AIDS patients without cryptosporidiosis[28].

AIDS patients infected with *C. parvum* also develop extraintestinal disease, most frequently of the biliary tract[29]. Biliary cryptosporidiosis has only recently been recognized as a clinical entity, and is even less understood than the intestinal form of the disease. Although a number of pathogens account for opportunistic biliary tract infections in AIDS patients, *C. parvum* is the single most common identifiable pathogen and is found in 20%-65% of the patients with so-called AIDS-cholangiopathy[9]. These patients often have right upper quadrant pain, nausea, vomiting, fever and biochemical evidence of cholestasis. Strictures, narrowing and irregularities of the intrahepatic bile duct s, with dilatation of the common hepatic and common bile ducts and the thickening of the ductal walls, are often found by noninvasive and invasive imaging studies[30].

Diagnosis

Indirect methods of diagnosing cryptosporidosis (by comparative symptomatology or clinical parameters) have proven unsatisfactory. Serological studies also have no place in diagnosis as many healthy individuals already have antibodies. At present, most cryptosporidial infections are diagnosed by the microscopic examination of host faecal material for the presence of *C. parvum* oocysts. Experimental studies have shown that oocyst excretion coincides well with the onset and duration of most clinical signs of disease. Most asymptomatic individuals can be screened to detect subclinical infections and even water samples can be examined for contamination by oocysts. *C. parvum* oocysts are much smaller than those of other coccidian parasites, and they differ in many of their staining and buoyancy characteristics. Thus, most conventional coprological techniques used in parasitology and microbiology laboratories are not entirely suitable for their detection. Many specialized staining procedures have been described to stain the oocysts and differential staining techniques are more desirable to avoid confusion. The technique of choice for many diagnostic laboratories has been acid-fast staining. Oocysts stain bright red whereas yeast, bacteria and other faecal debris only take up the counterstain. Several immunolabelling techniques have also been

developed to detect oocysts. Both polyclonal rabbit antisera and monoclonal antibodies have been used to detect *C. parvum* oocysts in faecal and water samples by immunofluorescence and several diagnostic test kits are now commercially available[19,31,32].

When biliary disease is suspected, ultrasonography is the best initial diagnostic method. It will be suggestive in most cases by identifying biliary ductal wall thickening and/or gallbladder dilation or both. Computerized tomography might also be helpful. However, the most sensitive method to diagnose biliary tract disease in HIV-infected patients is endoscopic retrograde cholangiopancreatography (ERCP)[30]. If biliary disease is highly suspected and the patient has normal ultrasonography, ERCP should be performed. However, ERCP is not recommended to work-up suspected asymptomatic AIDS-cholangiopathy. The cholangiographic a ppearance of AIDS cholangiopathy is quite variable and has been described in different ways. Characteristically, the biliary tree appears irregular and distorted with focal dilation and narrowing in the intrahepatic and/or extrahepatic biliary tree. The most common cholangiographic pattern is papillary stenosis associated with intrahepatic sclerosing cholangitis, which occurs in approximately 50% - 60% of patients[30,33,34]. Although occasionally diagnostic, percuta neous liver biopsy is rarely helpful and thus plays no role in the diagnosis of AIDS-cholangiopathy. Serum alkaline phosphatase is the most commonly elevated liver biochemical test with mean values in most series of 700 IU/L-800 IU/L. Mild increases in aminotransferases are common with values ranging from 65 to 123 IU/L, whereas hyperbilirubinemia is distinctly uncommon[30].

THERAPY AND PREVENTION

Therapy

At present, there is no effective antimicrobial treatment available for cryptosporidiosis in man or animals. Gererally, immunocompetent individuals need no specific therapy. Supportive care with oral or intravenous fluids and electrolyte replacement is beneficial in alleviating the dehydration accompanying acute diarrhea while awaiting spontaneous recovery. In children, however, spiramycin may shorten the duration of oocyst excretion and diarrhea, although conflicting results have been obtained[35,36]. In AIDS patients without antiretroviral therapy, AZT therapy should be started. In these patients, a relationship between disease severity and $CD4^+$ count has been documented. Paromomycin is the only agent so far that has been found to have efficacy in animals and humans in the treatment of intestinal crytosporidiosis[37-39]. It is an aminoglycoside anti biotic that is not significantly absorbed when given orally and is used for intestinal amoebiasis. In most studies, including a double blind trial, there was a good clinical and parasitological response[37-40]. However, after discon tinuation of treatment, many patients relapse. In a well-documented study, paromomycin, 500 mg 4 times daily for 4 weeks and maintenance therapy of 500mg twice daily, was used[41].

Therapy of biliary cryptosporidiosis in AIDS-cholangiopathy is primarily endoscopic[30]. For those patients with abdominal pain or cholangitis associated with papillary stenosis, endoscopic sphincterotomy may provide striking symptomatic relief as it facilitates drainage and decompression of the biliary system. Although survival is not prolonged, sphincterotomy may help improve the quality of life for those with papillary stenosis and pain. There is no evidence that sphincterotomy is beneficial for sclerosing cholangitis in the absence of papill ary stenosis and CBD dilation, or in asymptomatic patients and it may be associated with a higher complication rate. Patients with diffuse intra- and extrahepatic sclerosing cholangitis alone have few specific treatment options. Paramomycin is not effective in biliary cryptosporidiosis in AIDS-cholangiopathy[40].

Prevention

Since therapy remains difficult, prevention of the disease is critical. *C. parvum* infections are contracted by the ingestion or inhalation of oocysts, and therefore effective control measures must aim to reduce or prevent oocyst transmission. *C. parvum* have been proven remarkably resistant to chemical disinfection. Many commercial disinfectants (based on aldehyde, ammonia, alcohol, chlorine or alkaline compounds) are ineffective when used according to the manufacturers' instructions and most conventional methods of water treatment do not effectively remove or kill all the oocysts from contaminated water[1]. Many recommendations have been made for the prevention and control of infections in specific locations; such as hospitals, laboratories, and day care centers. These recommendations have basically involved managerial practices designed to minimize further host contact with sources of infection. Isolation of infected individuals, careful handing and disposal of biohazardous waste, and heat treatment (boiling) of suspect contaminated water before consumption are helpful.

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Expression of inducible nitric oxide synthase in human gastric cancer

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INTRODUCTION

Inducible nitric oxide synthase (iNOS) is an enzyme that catalyzes the formation of nitric oxide (NO) from L-arginine. iNOS expression and activity results in the production of high levels of NO[1]. The generation of physiological levels of NO is important for mucosal function and it also exerts a cytoprotecti ve effect on the gastrointestinal mucosa. However, increased iNOS expression has been observed in patients with chronic inflammatory diseases of the gastrointes tinal tract, such as ulcerative colitis[2,3], and gastritis[4] and it has been speculated that increased NO may induce DNA damage[5,6] and angiogenesis[7]. Nonetheless, the role of iNOS in human GI neoplasia is largely unkown. Previous studies have demonstrated increased iNOS expression in breast cancer[8,9], and increased iNOS activity and protein levels have been demonstrated in colorectal cancer[10] and adenocarcinoma of the esophagus[11]. However, to date, the role of iNOS in gastric carcinogenesis has not been elucidated.

MATERIALS AND METHODS

Gastric biopsies were obtained from individuals undergoing gastric endoscopy. Two or three mucosal biopsies were endoscopically obtained for histological study. One or two additional biopsies were obtained for mRNA isolation. The biopsies were snap frozen in liquid nitrogen and stored at -80 ℃. The samples used in this study were collected from tumor and a tumor free location in 6 gastric cancer patients, and 7 biopsies were obtained from the histologically normal gastric mucosa in corpus and/or antrum from healthy subjects. RNA was extracted using the RNA-zol B procedure. After completion of this extraction, RNA was separated on a 1.5% agarose gel and RNA was visualized by ethidium bromide staining. cDNAs were generated from one microgram of total RNA; it was denatured at 65 ℃ for 10 min and cooled on ice for 2 min. The RNA was reversely transcribed in a 20 μL final volume of 5x AMV RT buffer, $MgCl_2$, dNTPs, random primers, 16 U of Rnasin and 1.5 U AMV Reverse Transcriptase. The reaction mixture was incubated for 1 hour at 37 ℃, and for 5 min at 96 ℃. For confirmation of cDNA integrity, a RT-PCR analysis using β-actin primers was also performed. The sequence of the primers were as follows: sense primer (s-iNOS), 5' TAGAGGAACATCTG-GCCAGG-3'; antisense primer (as iNOS), 5'-TG-GCAGGGTCCCCTCTGATG-3'; generating a 372 bp fragment of the iNOS transcript. PCR was performed under the following conditions: 94 ℃ for 5 min, 60 ℃ for 45 sec, 72 ℃ for 1min; which was repeated for 35 cycles. Ten mL of the PCR reaction was separated on a 1.5% agarose gel and cDNA was visualized by ethidium bromide staining.

RESULTS

RT-PCR analysis using primers specific for human iNOS mRNA generated a 372 bp fragment of the predicted size. Using this RT-PCR analysis iNOS mRNA was detected in 3 of 6 tumor tissues, and in one of the adjacent tumor free gastric tissues obtained from gastric cancer patients (Table 1). In addition, a fragment of iNOS mRNA was amplified in one of 7 normal gastric tissues obtained from four healthy individuals undergoing endoscopy (Table 2). *H. pylori* infect ion was detected histologically in 5 of 6 cancer patients and in the stomach of two of the four healthy individuals. In two of the *H. pylori* infected indivi duals iNOS mRNA was detected in the non-cancerous mucosa, whereas all individuals without *H. pylori* infection did not exhibit iNOS mRNA.

Table 1 iNOS expression in gastric cancer patients

Patient	Age	Sex	Cancer type	Hp status	iNOS expression	
					Tumor	Tumor-free
1	37	m	Intestinal	+	-	-
2	55	m	Diffuse	+	-	+
3	69	f	Diffuse	+	+	-
4	71	m	Intestinal	+	+	-
5	73	m	Unknown	+	+	-
6	63	m	Intestinal	-	-	-

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Table 2 iNOS expression in healthy individuals

Patient	Age	Hp	Gastritis	iNOS expression	
				Antrum	Corpus
1	24	-	+	-	-
2	37	+	+	-	+
3	60	+	++	-	nd
4	49	-	-	-	-

DISCUSSION

H. pylori infection of the gastric mucosa may lead to chronic gastritis[12]and to the development of gastric or duodenal ulcers[13]. Furthermore, *H. pylori* infection is considered a risk factor for gastric cancer[14-16]. The molecular alterations underlying the pathogenesis of gastric cancer, however , remain largely unkown. In addition, the molecular alterations induced by *H. pylori* infection of the gastric mucosa which may contribute to gastric carcin ogenesis are not well established. Recently several studies have identified high levels of iNOS expression in *H. pylori* associated gastritis[17,18]. Furthermore, it has been shown that both whole *H. pylori* bacteria and lysates may induce iNOS mRNA levels and iNOS release[11]. Interestingly, after eradication of *H. pylori* infection iNOS expression reverts as determined by immunohistochemistry[18]. In our present study we found that iNOS expression was present only in individuals infected with *H. pylori* infection, whereas individuals without *H. pylori* infection did not exhibit iNOS mRNA in the gastric biopsies.

The chronic inflammation caused by *H. pylori* may induce molecular and cellular pathways contributing to the malignant transformation of the gastric mucosa. In our study 4 of the 6 cancers exhibited iNOS mRNA. While the increased formation of NO may lead to DNA damage, may stimulate angiogenesis, and may inihibit DNA repair mechanisms, the increased expression of iNOS in gastric cancers raisesthe hypothesis that the chronic inflammation caused by *H. pylori* infection may lead to molecular alterations of the gastric mucosa which activate molecular pathways that could lead to the transformation of the gastric mucosa and the development of gastric cancer[19].

In summary, our study supports the hypothesis that molecular alterations induced by *H. pylori* infection of the gastric mucosa may precede the development of gastric cancer and provide a further link between chronic inflammation and malignant transformation in the gastrointestinal tract.

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Partial sequencing of 5'non-coding region of 7 HGV strains isolated from different areas of China

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Subject headings hepatitis G virus; polymerase chain reaction; nucleotide sequence; RNA, viral

INTRODUCTION

Although sensitive tests for detection of known hepatitis viruses are available, the etiology of 10%-15% post-transfusion and community-acquired hepatitis cases has remained undefined. It suggests the existence of unknown causative agents associated with the disease. GBV-C and HGV were newly discovered as putative non-A to E hepatitis viruses reported by Simons[1] and Linnen[2] independently. However, the sequence homology analysis of the two strains revealed that they are different isolates of the same virus. HGV is a positive-strand RNA virus with an entire genome of 10kb which contains a continuous open reading frame (ORF) encoding a viral polyprotein. The structural region (C, E1 and E2) is located at the N-terminal, while the non-structural region (NS2, NS3, NS4A/B, NS5A/5B) is situated at the C-terminal. The long ORF is preceded by a 5' untranslated sequence and followed by a 3' untranslated sequence. Our previous report has confirmed the existence of HGV infection in China[3]. There is evidence that the gene of hepatitis C virus (HCV) is hypervariable in different areas[4-8]. The variability of HCV is also found in the same strain of the virus. HGV and HCV are classified in the same genus of the flaviviridae family. So it is of great significance to clarify the geographical distribution of HGV genotypes in the world[9]. In this study, the partial sequences of 5' non-coding region of 7 HGV strains isolated from different areas of China were analyzed and compared with GBV-C (U36380) and HGV (U44402) reported from the United States.

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MATERIALS AND METHODS

Subjects

Seven HGV RNA positive sera tested by RT-PCR were collected from blood donors of Beijing, Jiangsu, Anhui, Liaoning, Hebei Provinces, and Guangxi Zhuang and Xin jiang Uighur Autonomous Regions.

Primers

According to the nucleotide sequence of 5' non-coding region of a Chinese HGV strain, the primers for RT nPCR were designed using the software of OLIGO 5.0. They were as follows: S1 5' GGT GGT GGA TGG GTG ATGAC 3'; A1 5' CCG AAG GAT TCT TGG GCT AC 3'; S2 5' GCT GGT AGG TCG TAA ATC 3'; A2 5 ' ACT GGT CCT TGT CAA CTC 3'.

Detection of HGV RNA and nucleotide sequencing

HGV RNA extraction, HGV cDNA synthesis and PCR procedure were performed by the methods described previously[3]. All the PCR products were cloned into the pGEMT vector (Promega, Madison, WI), and positive clones were identified. The PCR products were purified and sequenced bidirectionally using the dideoxynucleotide chain termination method. The HGV cDNA sequences were analyzed with a DNA sequencer (ABI PRISM 377 DNA Sequencer, Perkin-Elmer Cetus).

RESULTS

Detection of HGV RNA

The positive rates of anti-HGV varied from 1.2% (35/2916) to 5.4% (49/907) in blood donors and 42.9% (15/35) 75.5% (37/49) of anti-HGV positive sera were also HGV RNA positive.

Partial sequencing of 7 Chinese HGV strains

The partial nucleotide sequences of the 5' non-coding region of 7 HGV strains isolated from blood donors of Beijing, Jiangsu, Anhui, Liaoning, Hebei Provinces, and Guangxi Zhuang and Xinjiang Uighur Autonomous Regions, China were analyzed and compared with GBV-C (U36380) and HGV (U44401) (Figure 1).

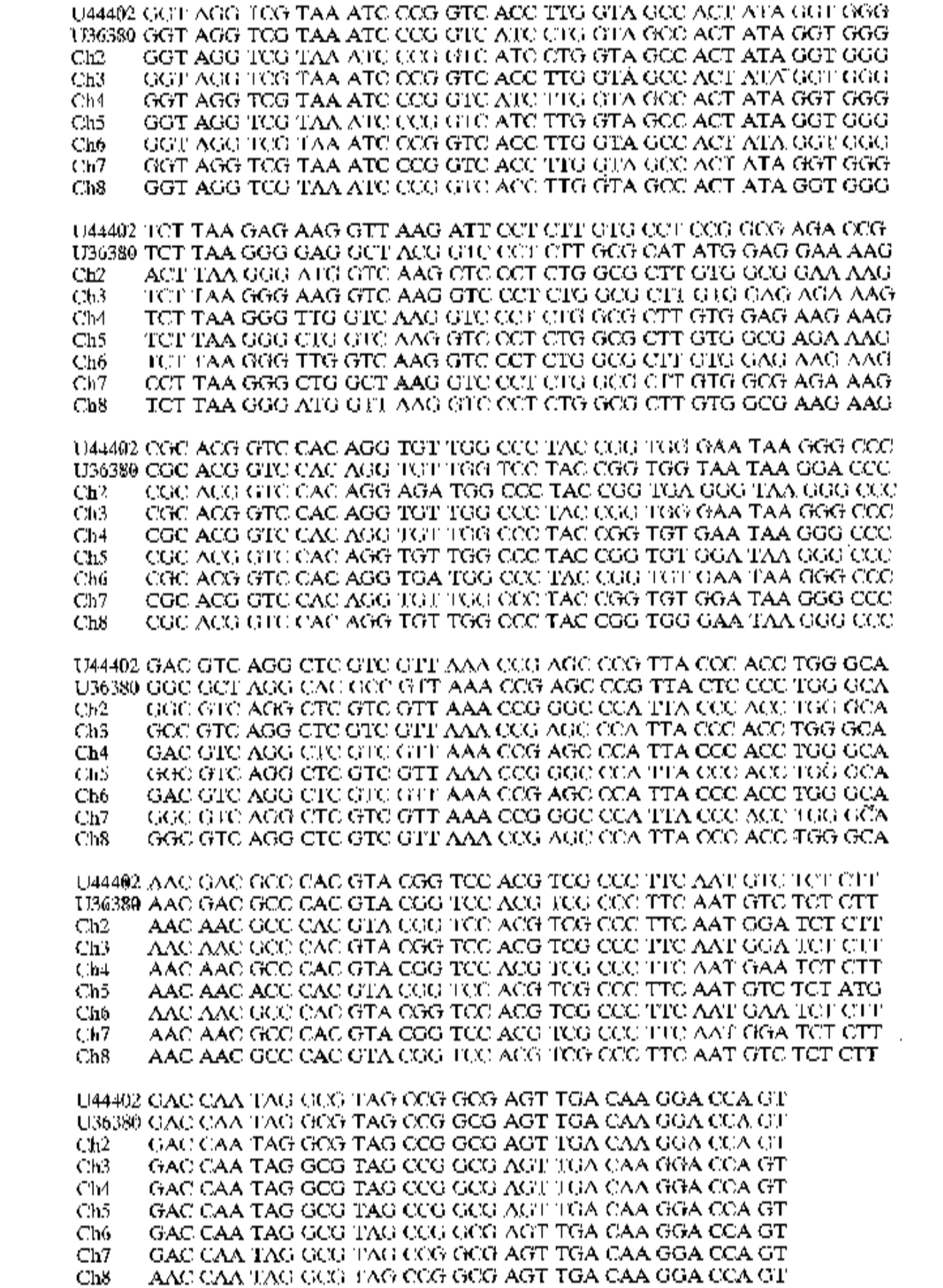

Figure 1 Comparison of partial nucleotide sequences of the 5'non-coding region of 7 HGV strains isolated from different regions of China. Ch2: Beijing; Ch3: Jiangsu; Ch4: Anhui; Ch5: Liaoning; Ch6: Guangxi; Ch7: Xinjiang; Ch8: Hebei

Table 1 Comparison of the partial nucleotides of 7 Chinese strains o f HGV with reported strains

HGV strains	Homology of the nucleotides (%)								
	U36380	U44402	Ch2	Ch3	Ch4	Ch5	Ch6	Ch7	Ch8
U36380	100.0								
U44402	87.23	100.0							
Ch2	85.92	86.85	100.0						
Ch3	88.26	92.02	93.42	100.0					
Ch4	88.26	86.67	92.96	96.24	100.0				
Ch5	85.54	89.20	92.96	93.90	94.37	100.0			
Ch6	86.85	89.67	92.96	96.24	99.06	93.43	100.0		
Ch7	85.92	89.67	94.84	97.18	95.31	96.71	95.31	100.0	
Ch8	88.26	91.55	92.02	95.30	95.31	93.70	95.31	94.37	100.0

Homology of 7 Chinese HGV strains

The nucleotide homology of the 5' non-coding region of 7 Chinese HGV strains was 85.92%, 88.26%, 88.26%, 85.45%, 86.85%, 85.92% and 88.26%, respectively, as compared with the African strain GBV-C (U36380). It was 86.85%, 92. 02%, 86.67%, 89.02%, 89.67% and 91.55%, respectively, as compared with the A merican strain HGV (U44402). The homology of nucleotide sequences was 92.02 % - 97.18 % among the 7 Chinese HGV strains (Table 1).

DISCUSSION

HGV is transmitted parenterally, and the infection seems not to cause significant hepatic damage as hepatitis viruses A E do. Although transmission through blood or parenteral exposure is well documented for HGV, little is known about its prevalence in blood donors of China. This study shows that the prevalence rate o f anti-HGV ranged from 1.2% to 5.4% in the population of different areas of C hina. The data indicate that the HGV infection is widely spread in the different areas of China. The nucleotide homology of the 5' non-coding region among the 7 Chinese HGV strains was 92.0%-97.2%. However, the identity of these 7-Chinese strains was 85.9 % - 92.0 % at the nucleotide level as compared with the African strain of GBV-C (U36380) and the American HGV strain (U44402). The data suggest that the Chinese HGV isolates belong to a new group which is different from the African and American strains reported by Simons[1] and Linnen[2]. The divergence of nucleotide sequences among Chinese HGV strains shows the correlation between HGV variation and the geographical locations.

The homology of NS3 nucleotide sequences of the 3 Chinese HGV strains reported previously by our group[3] was 92.48%, 89.09% and 85.34%, respectively with GBV-C (U36380), and 89.09%, 85.34% and 85.34% with HGV (U44402). It is very close to the homology of the 5'non-coding region of 7 Chinese HGV strains with GBV-C (U36380) and HGV (U44402), indicating that the NS3 region may not be the site of immune selection.

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Apoptosis of neoplasm cell lines induced by hepatic peptides extracted from sucking porcine hepatocytes

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Subject headings neoplasm cell lines; apoptosis; hepatic pepti des; hepatic extracts; liver neoplasms; hepatocytes

INTRODUCTION

Promoting hepatocyte growth factor (pHGF) extracted from the sucking pig liver is a series of polypeptides with molecular weight less than M_r 10 000 and has specific biological activities to stimulate the rat hepatocyte DNA synthesis after 1/3 partial hepatectomy and promote recovery of rat hepatic injuries induced by endotoxin and D-aminogalatose. These properties are similar to the results reported by Labrecque[1]. pHGF can effectively cure clinical acute fulminant hepatitis, chronic hepatitis and other hepatic injuries by significantly reducing serum alanine aminotransferase (sALT), eliminating jaundice and increasing the survival rate of fulminant hepatitis[2,3].

It was reported that three of the six fractions of pHGF purified by HPLC, can promote the DNA synthesis of rat primarily cultured hepatocytes[4,5]. Two of the fractions can inhibit the proliferation of BEL-7402 hepatoma cell line, their activities were not cross- affected, indicating that there exist at least two different active components in pHGF. To investigate the inhibitory mechanism of pHGF, we purified these inhibitory components and studied their physico-che mical properties and apoptosis inducing effects.

MATERIALS AND METHODS

Primarily cultured hepatocytes

Male Sprague Dowley rats of 250g±30g in weight were purchased from the Experimental Animal Center, Sun Yatsen University of Medical Sciences, Guangzhou, China. The animals were anaesthetized with ether, and their livers were removed, decapsulated, cut into small pieces, rinsed with PBS (pH 7.4) to wash away the blood. The liver pieces were incubated with 0.25% trypsin (Gibco) for 30min and gently homogenized. The hepatocytes were collected after centrifugation at 1000×*g* for 10min. Pellet parenchyma cells were washed three times with PBS (pH 7.4) by centrifugation, resuspended in RPMI-1640 medium containing 10 % FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin and placed to Costar (3596) 96 well culture plate at a density of 10.6 cells/ cm^3, 0.1mL each well. The cells were cultured at 37 ℃, 5% CO_2 until use.

Neoplasm cell lines

BEL-7402 human hepatoma cell, Hepe murine hepatoma cell and CNE-2 human nasopharyngeal carcinoma cell lines were gifts of Experimental Animal Center, Sun Yat -sen University of Medical Sciences. SMMC-7721, QGY-7703 human hepatoma cell lines, HCT_8 human colic adenocarcinoma cell and GLC-82 human lung adenocarcinoma cell lines were purchased from the Cellular Institute of Chinese Academy of Sciences. SGC-7901 human gastric carcinoma cell line was purchased from the Biochemistry Department of the Fourth Military Medical University. The cultured cells were grown to confluence, digested with 2.5 g/L trypsin containing 0.2 g/ L EDTA, washed with PBS (pH 7.4) and resuspended in DME/F12 or RPMI-1640 medium, added into Costar (3596) well culture plates at a density of 2.5-5.0×10^4 cells/mL in 0.05 mL each well, then incubated at 37 ℃, 5% CO_2 for 24 h. After remained at 4 ℃ for 1 h, the cells were immediately recovered at 37 ℃ for further use.

Proliferation inhibitory experiments

Various concentrations of HP (S4) in DME/F12 or RPMI-1640 medium containing 10% FCS were added to the cultures, incubated for another 12, 24, 48 and 72 h, MTT solution (1.5 g/L in PBS) was then added to all wells (10 μL per 100 μL medium) in the last 4 h-6 h, rinsed with PBS, DMSO was added and vibrated to dissolve the dark blue crystals. After placed at room temperature for a few minutes until all crystals were dissolved, the optic-metric density (OD) was read on Σ960 ELISA

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reader at test wavelength of 570 nm and referent wavelength of 630 nm.

Apoptosis inducing experiments

In situ cell death detection kits (POD) were purchased from Oncor Co., USA. The cells were adjusted to a density of 2.5 - 5.0×10^4 cells/cm^3, added to 24-well plates with cover glass-slides in 0.5 mL each well. Incubated at 37 ℃ 5% CO_2 for 24 h, the cells were remained at 4 ℃ for 1h, the tempera ture was then promptly recovered to 37 ℃. Various concentrations of S4 were added and incubated for different periods. The glass slides were taken out, rinsed, fixed and immunohistochemically stained. The negative control with omission of Tunel enzyme was designed according to the manufacturer's manual. The cells stained with dark yellow brown nucleus were considered as positive cells. Ten optical fields, about 500-1 000 cells were counted in each glass-slide under the high magnification (× 400) microscope. Results presented as ± indicated 5% slightly positively stained cells; +, >5 <10% positive cells; + +, >10 <20% positive cells; and +++, >20 <30% positive cells.

Effects of S4 on the expressions of 4 oncogenes in 8 neoplasm cell lines

The antibodies used in this study including p53, a murine monoclonal antibody IgG2b against human p53, and Bcl-2, a murine monoclonal antibody against human Bcl-2 -p25 oncoprotein were purchased from DAKO Co Inc. Fas, a rabbit polyclonal antibody against human Fas oncoprotein, and c-myc, a murine monoclonal antibody IgG2a against p67 oncoprotein purchased from Santa Cruz Biotechnology Inc, UK. Secondary antibody (DAKO products) was a rabbit antibody against murine immunoglobulin conjugated with horseradish peroxidase.

The neoplasm cells were adjusted to 2.5-5.0× 10^4cells/cm^3, added into 24 -well culture plates with cover glass-slides in 0.5 mL each well, incubated at 37 ℃ 5% CO_2 for 24 h, then remained at 4 ℃ for 1 h for cell growth at the same step. Immediately after the temperature was recovered to 37 ℃, S4 was added and continued to inoculate for 48 h, then the cells were fixed and immunohistochemically stained following the instruction of the manufacturer. The negative control with primary antibody was replaced by PBS, and observed under light microscope. The results were presented as "+ +" to "+ + +" which indicated the slight positive staining to the strongest positive staining.

Northern blot

The quantity of cells was adjusted to 1×10^7 per dish, and RNA in the cells was extracted rapidly with Guanidinium isothiocyanate. According to the method of random primer (Promega), the cDNA probe of human Bcl-2 gene was labeled with α-^{32}P-dCTP, and hybridized for 24 h at 68 ℃ in 6×SSC, 2 ×Denbart's reagent and 0.5% SDS. After the filters were washed, autorad iography was performed by exposing the filter to X-ray film at -70 ℃ for 48 h. The results were determined with an intensifying screen.

Apoptosis of BEL-7402 hepatoma cells transplanted in BALB/C nude mice induced by S4

Male BALB/C nude mice, weighing 18 g-22 g were provided by the Experim ental Animal Center of Sun Yat sen University of Medical Sciences. BEL-7402 he patoma cells were injected s.c. into the right neck (1.46×10^7 cells/mL) of BALB/C male nude mice. When growing to about 0.8 cm^3, tumors were cut into small pieces of about 1 mm^3 and transplanted into the renal capsulae of BALB/C nude mice, and their width and lengths were measured with micrographer. Twenty-six mice were divided into four groups. In the high-dose group, the six mice were injected ip with 1.0 mg S4/kg-per day and in the low-dose group, with 0.5 mg of S4/kg per day. In the positive control group, the six mice were injected ip with 1 mg Doxorubicin (Huaming Pharmaceutical Co, LTD)/kg per day, while in the negative control group, the eight mice were injected ip with 0.4 mL physical saline daily for 16 days. The nude mice were killed 2 days after the end of treatment. The width and lengths of the tumors were measured with a micrographer. All the data including body weight of mice were input to computer to calculate the growth inhibitory rate of tumors and T value.

Statistical analysis

Statistical significance was determined by using χ^2 test or Student's *t* test.

RESULTS

Homogeneous peak of hepatocyte extracts

DEAE-Sephadex A25 purified hepatocyte extract was desalted and lyophilized, the proliferation inhibitory activities were tested. The active fraction was further purified by Superdex 75 and C_{18} reversed-phase chromatography and lyophilized. The last purified fraction was called Subfraction 4 (S4). A combination of the above procedures produced an almost pure peak with 95.8% relative area, and molecular weight of M_r 4020.6 determined by HPLC and MALDI-TOF-MS mass spectrometer.

Proliferation inhibitory effect of pHGF and its

fractions on normal hep atocytes and 7402 hepatoma cells

After the hepatocytes were removed from the livers of the SD rats, 7402 hepatoma cells were incubated for 24 h, 500, 1 000 and 2 000 mg/L of pHGF and 20 mg/L S4 purified by DEAE-Sephadex A25 were added into each well with 0.1mL medium. After incubation at 37 ℃, 5% CO_2 for 48 h, MTT was added and reincubated for 4 h-6 h, then DMSO was added and mixed thoroughly to dissolve the dark blue crystals. OD value was read on ∑960 ELISA reader. The inhibitory rate (%)=(100 test group OD value/control group OD value ×100%). pHGF apparently inhibited the proliferation of BEL-7402 hepatoma cells at concentrations of 500 mg/L - 2 000 mg/L, and promoted the activities of primarily cultured hepatocytes. Of six fractions of pHGF purified by DEAE- Sephadex A25, fractions 3 and 4 significantly inhibited the growth of 7402 hepatoma cells while fractions 1, 2 and 5 promoted the activities of primarily cultured hepatocyte dehydrogenase (Table 1).

The results showed that the fractions of pHGF both promoted the activities of normal hepatocyte dehydrogenase and inhibited the activities of hepatoma cell dehydrogenese. There were no cross effects between the two actions.

Inhibition of S4 on the proliferation of 8 neoplasm cell lines

S4 at concentrations of 1, 5, 10 mg/L were added into the culture plates (96 wells), each well containing 0.1 mL medium, and incubated at 37 ℃, 5% CO_2 for 12, 24, 48 and 72h. MTT solution (1.5 g/L in PBS) was added to all the wells (10 μL per 100 μL medium), and incubation was continued for h - 6 h, then 100 μL DMSO was added and mixed to dissolve MTT dark blue crystals. After vibrated at room temperature for 15min, the plates were read on ∑960 ELISA reader at test wavelength of 570 nm and referent wavelength of 630 nm. The results showed that S4 can significantly inhibit the proliferation of the 8 neoplasm cells with a clear dose and time dependent manner (Table 2).

Apoptosis of 8 neoplasm cells induced by S4

Based on the above results that S4 can induce tumor cells to die at concentrations of 1 mg/L-10 mg/L from 24 to 72 h, we selected the 5 mg/L of S4 and 48 h affecting period as experimental conditions so as to better compare the results of S4 on 8 neoplasm cell lines. The apoptosis inducing effect of pHGF and S4 on BEL-7402 hepatoma cells showed that the apoptosis inducing effect of S4 is 100 times as strong as that of pHGF calculated by their activities in weight. S4 induced apoptosis of 8 neoplasm cell lines with different activities, and apparently induced all the hepatoma cells to die, the effect on non-hepatoma cell lines being smaller than that of hepatoma cell lines. S4 had no apparent apoptotic effect on HCT-8 cell line, and even stagnated the apoptosis of CNE-2 cell line (Table 3). All these may be related to the histocellular sources and the signal differences tran smitted in the cells.

Effect of S4 on the 4 oncoprotein expressions of 8 neoplasm cell lines

Confluent cells digested by trypsin, adjusted to 2.5-5.0×10^4 cells/cm^3, added into 24 - well Costar plates with cover glass-slides in each well with 0.1mL medium and cultured in DME/F12 medium containing 10% FCS, at 37 ℃, 5% CO_2 for 24 h. To grow at the same step, the cells were placed at 4 ℃ for 1h before use. Temperature was quickly recovered to 37 ℃, 5 mg/L S4 was added and incubated for an other 48 h. Immunohistochemistry staining was performed following the manufacturer's manual and positive and negative controls were designed. The results (Table 4) showed that S4 significantly up -regulated the expression of P53, Fas and depressed Bcl-2, but had no apparent effect on the expression of c-myc in 4 hepatoma cell lines, and the most prominent effect fell upon the BEL-7402 cell line. Effects of S4 on regulating oncogene expressions of 4 nonhepatoma neoplasm cell lines differed. S4 slightly promoted the expression of Fas and depressed Bcl-2 and c-myc in 7901 cell line; slightly down-regulated Bcl-2 and c-myc in GLC-82 cell line; but promoted the expression of Bcl-2 in CNE-2 cell line. Based on the results in 8 neoplasm cells, the apoptotic induction of S4 on the cells was considered to be exerted via affecting the Bcl-2 oncogene expression.

Table 1 Effect of pHGF and its fractions on normal hepatocytes an d hepatoma cells ($\bar{x}$±s, *n* = 8)

	pHGF (OD, mg/L)			Fractions (OD, 20mg/L)						Control
	500	1000	2000	1	2	3	4	5	6	
7402 cells	0.422±0.11[b]	0.410±0.09[b]	0.401±0.07[b]	0.545±0.03	0.601±0.11	0.427±0.067[b]	0.411±0.09[b]	0.580±0.08	0.649±0.21	631±0.12
Primary hepatocytes	0.390±0.07[b]	0.398±0.09[b]	0.388±0.06[b]	0.293±0.06[b]	0.308±0.06[b]	0.223±0.07	0.236±0.06	0.280±0.10[b]	0.219±0.11	0.218±0.07

[b]P<0.01, *vs* control.

Table 2 Effects of S4 on 8 neoplasm cell lines at different time points

Cells	IC50 (mg/L)		
	24h	48h	72h
BEL-7402	8.2	5.0	3.9
SMMC-7721		8.4	4.2
QGY-7703		24.2	9.5
Hepe	15.2	8.9	6.5
SGC-7901	30.2	19.7	14.3
GLC-821	2.4	9.9	5.4
CNE-2			8.9
HCT-8			9.9

IC_{50}: the half inhibitory concentration.

Table 3 Apoptosis of 8 neoplasm cell line induced by S4 (5mg/L, 48h)

	Control	S4 Treatment
BEL-7402	+	++
SMMC-7721	+	++
QGY-7703	±	++
Hepe	+	++
HCT-8	±	±
GLC-82	±	+
SGC-7901	±	+
CNE-2	++	+

Table 4 Effects of S4 on the 4 oncogene expressions of 8 neoplasm ce ll lines (S4, 5μg/well, 48h)

	P53		Fas		Bcl-2		c-myc	
	C	T	C	T	C	T	C	T
BEL-7402	+	++	+	++	+	±	+	+
SMMC-7721	+	++	+++	+++	+	±	++	++
QGY-7703	±	+	+	+++	±	±	+	++
Hepe	+	++	++	++	++	±	++	+
HCT-8	+	+	++	++	±	±	++	+
GLC-82	++	+	+++	+++	+	±	++	+
SGC-7901	+	+	±	+	+	±	++	+
CNE-2	+	++	+++	++	±	+	++	+

C: control group; T: test group.

Northern blot

The changes of Bcl-2 mRNA in BEL-7402 cell line after incubation with 5 mg/ L S4 were observed by blot hybridization. The results showed that S4 can apparently inhibit the Bcl-2 mRNA transcription determined by the quantity measure ment of intensifying screen. Relative value of the test group was related to the dose of S4. This result was concordant with the oncoprotein expression and demo nstrated that S4 can inhibit the Bcl-2 oncogene expression at mRNA transcript level.

Experiments of BALB/C nude mice transplanted with BEL-7402 hepatoma cells

The mice transplanted with BEL-7402 hepatoma cells were injected ip with S4 at d3 after tumor transplantation, once daily for 16 days. The results showed that S4 can apparently inhibit the growth of BEL-7402 hepatoma cells innude mice. This inhibitory effect was also induced by apoptosis (Tables 5 and 6). S4 had no apparent effect on the mitosis and differentiation of BEL-7402 hepatoma cells observed under the optical microscope. Cell shrinkage and condensation, microvillus disappearance, condensed chromatin margination and apoptotic body formation were found under electronic microscope.

Table 5 Inhibition of S4 on hepatoma cells in nude mice ($\bar{x}\pm s$, n=6)

	Dose (mg/kg)	Do (mm^3)	Dn (mm^3)	Dn-Do (mm^3)	Inhibitoryrate (%)
High dose of S4	2.0	2.19±0.24	2.81±0.49	0.61±0.38	59[b]
Low dose of S4	1.0	2.00±0.12	2.55±0.22	0.56±0.26	63[b]
Doxorubicin	1.0	2.26±0.12	2.16±0.32	0.10±0.29	107[b]
Control	0.0	2.17±0.23	3.67±0.52	1.50±0.55	

Do: Dimension of tumors at onset; Dn: Dimension of tumors at necropsy. [b]P<0.01, *vs* control.

Table 6 Apoptosis of hepatoma cells in nude mice induced by S4 (LI, $\bar{x}\pm s$, n=6)

	Dose (mg/kg)	LI (%)
High dose of S4	2.0	18.70±4.92[b]
Low dose of S4	1.0	13.22±1.74[b]
Doxorubincin	1.0	14.18±2.46[b]
Control	0.0	8.40±2.81

[b]P<0.01, *vs* control. LI: lable index.

DISCUSSION

There are lots of substances in the human body which can stimulate the proliferation and regeneration of liver, e.g. insulin, platelet derived growth factor (PDGF), transfer growth factor β (TGF-β), epithelial growth factor (FGF), etc, but their effects are not specific. It has been shown that the hepatocytes of young animal livers might produce network self-regulators to modu late their growth, differentiation and apoptosis. Because of the differences in extracting and preparing the liver derived growth stimulating factors and inhibitors, it is difficult to enunciate whether these stimulating or inhibitory effects are caused by the double or poly-functional effects of one factor or by the specific effects o f two factors with different functions.

It was reported by Labrecque[1] that a series of substances extracted from the regenerating livers of young rats, dogs and rabbits with M_r12 000-21 000 are called hepatic stimulating substance (HSS) that can stimulate the DNA synthesis of the primarily cultured hepatocytes and the hepatocytes after partial hepatectomy (2/3)[1]. HSS can raise the survival rate of rats with fulminant hepatic failure and inhibit the growth of some neoplasm cell lines.

A series of polypeptides which can raise the survival rate of human fulminant he patitis, reduce sALT, eliminate jaundice and ameliorate liver functions are called promoting hepatocyte growth factors (pHGF). The monoclonal

immunohistochemical study showed that pHGF is located in the hepatocytes, not in mesenchymal cells such as Kupffer cells, endotheliocytes and fibroblasts[6].

In our previous papers[4], we found that pHGF contained not only hepatic regeneration stimulating substances, but also growth inhibitory factors which can inhibit the growth of hepatoma cell lines. These inhibitory factors can not inhibit the growth of normal primarily cultured hepatocytes. Homogeneous fraction S4 extracted from sucking pig liver by HPLC and FPLC in this report is a small peptide with M_r4020. Its inhibitory activities are 100 times as that of pHGF and can be destroyed by pronase K, but resist to acids, alkalis, RNAase and DNAase.

We selected 8 neoplasm cell lines to study the biological activities of S4 in the growth inhibition, apoptotic induction and oncogene expression related with apoptosis of these cell lines *in vitro*, and studied the effects of S4 in the hepatoma cells transplanted in nude mice *in vivo*. The results showed that S4 inhibit the proliferation of neoplasm cell lines *in vitro* by inducing apopto sis in a clear dose and time dependent manner. The apoptotic effect of S4 on the se cells is exerted via regulating the expression of oncogenes related to apoptosis, especially by affecting Bcl-2 gene expression. It was testified by *in vitro* and *in vivo* tests that S4 is an effective apoptotic inducer of neoplas mcell lines.

Modern molecular biology investigations have indicated that proliferation inhibition of some neoplasm cells is related to apoptosis induction regulated by the oncogene expression of these cells. The different growth inhibitions of S4 were found in 8 neoplasm cell lines with the different expressions of p53, Fas, Bcl-2 and c-myc oncogenes. S4 can apparently promote the oncoprotein expression of p53, Fas and depress the Bcl-2 and c-myc oncogenes while inducing hepatoma cells to die. It was reported that p53 induced apoptosis via stimulating apoptosis inducing genes, and inhibiting the survival necessitating genes, or participating in the enzyme cleavage of DNA. The apoptotic induction of Fas is caused by its ligands and receptor reaction, and the apoptotic induction of c-myc is related to the apoptotic mechanism controlled by Bcl-2 gene, which depends on the circumstantial factors, leading to either death or proliferation[7,8].

It is generally considered that Bcl-2 is an important gene for cells to survive, we have further confirmed that the Bcl-2 gene expression of BEL-7402 hepatoma cells is inhibited by S4 at m-RNA transcript level by using the techniques of blot hybridization. This may be the initial factor for the cell apoptotic induction, because Bcl-2 gene can inhibit the apoptosis by blocking the last tunnel of apoptotic signal transmitted system.

S4 inhibited the growth of hepatoma *in vivo* by inducing apoptosis, and had no apparent effect on the mitosis and differentiation of hepatoma cells. The results showed that the apoptotic induction of S4 is directly working against the target cells.

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The possible relationship between hepatomegaly and release of HGF into plasma induced by clofibrate in rats

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Subject headings clofibrate; hepatocyte growth factors; hepa tomegaly; rat

INTRODUCTION

Hepatocyte growth factor (HGF) is a newly discovered multifunctional growth factor, and the most potent mitogen known for hepatocyte[1]. It has been shown that recombinant human HGF can protect animal liver against the damaging action of carbon tetrachloride[2]. However, the generation of HGF is also associated with certain liver diseases and several extra-hepatic diseases[3-5]. For example, after partial hepatectomy or carbon tetrachloride poisoning, HGF level in rat plasma increases markedly[3,4]. Therefore, it is believed that HGF plays an important role in the restoration and regeneration of liver after injury.

As hypolipidemic drugs, such as clofibrate, may cause experimental hepatomegaly, proliferation of hepatic peroxisomes and hepatic carcinoma, they are taken as a unique class of carcinogens[6-8]. How the HGF level changes after liver damage due to clofibrate or the like has not been reported. This study was designed for examination of the relation between hepatomegaly and the changes of plasma HGF levels in rats caused by clofibrate.

MATERIALS AND METHODS

Animals

Male Wistar and Sprague-Dawley rats were supplied by the Laboratory Animal Center of the First Military Medical University.

Clofibrate administration

Clofibrate (bought from Shanghai 19th Pharmaceutical Factory) was given at doses of 500, 400, 300, 200 or 100mg·kg^{-1}·d^{-1} by adding it into food.

Measurement of liver weight

At different time intervals after clofibrate administration, the rats were killed with their livers weighed, and the weight was expressed as the percentage of body weight.

Serum preparation

At different time intervals after clofibrate administration, the tail of the rat was severed with a knife and the whole blood was collected. After centrifuge, the serum was stored at -20 ℃ in a freezer.

Measurement of the biological activity of HGF in the primary culture of rat hepatocytes[9]

Isolation of rat hepatocytes Male Sprague-Dawley rats were supplied by the Laboratory Animal Center of the First Military Medical University. The rats were 6-7 weeks old, weighing about 200 g. They were fasted for 8 h-10 h before operation, then were anesthesized with ether. Ane sthesia was discontinued when the respiration turned from rapid and shallow to slow and deep. The rats were fixed on a wooden plate, and the whole abdomen was sterilized with 750 mL/L (V/V) alcohol. The peritoneal cavity was cut open along the midline, and the portal vein and the inferior vena cava were exposed accordingly. The distal part of the portal vein was ligated, and a cannula was inserted into the proximal portal vein. D-Hank's solution (preheated to 37 ℃) was used for the infusion of liver, and at the same time, the inferior vena cava was cut open for blood-letting. The rate of infusion was about 30 mL·min^{-1}-40 mL·min^{-1} from slow to rapid to wash out any stagnant blood from the liver. After infusion, the surface of liver became smooth and wet, and light-yellow in color. The infusion fluid was then changed to 0.3 g·L^{-1} collagenase solution. Small vacuoles formed slowly on the connective tissue membrane of the liver surface with gaps in the liver tissue under the vacuoles. Eye-forceps were used to press lightly on the surface, and when the pressure was relieved, the infusion was stopped. The capsule of the liver was then torn open very carefully, and the liver tissue was put into Ham-F12

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hepatocyte culture medium (preheated to 37 ℃), and was blown mildly with a pipette into dispersed free liver cells, which were then filtered through nylon nets of 200 and 400 mesh size, and were centrifuged with 50 ×g three times at 4 ℃. Thus, the hepatocytes with more than 90% viability could be obtained. The cell density was adjusted to 1.5×10^5/mL, and the cells were plated in a 96-well culture plate at 200 uL per well. These liver cells were incubated in 50 mL/L CO_2 for 24 h. After the cells adhe red to the vessel wall, the culture media were replaced by serum-free ones and incubated for another 24 h. Afterwards, ^{3}H-TdR (185 MBq·L^{-1}) and the animal serum to be tested (which was diluted to different concentrations) were added and incubation continued for 24 h. Standard recombinant human HGF (to replace the animal serum) was used as the positive control. After incubation, the cells were harvested with a multi-channel cell collector and cpm values were measured with a liquid scintillation counter (Beckman Co., USA, Model LS9800).

RESULTS

The variation of HGF level in rat serum 4 days after clofibrate adminis tration

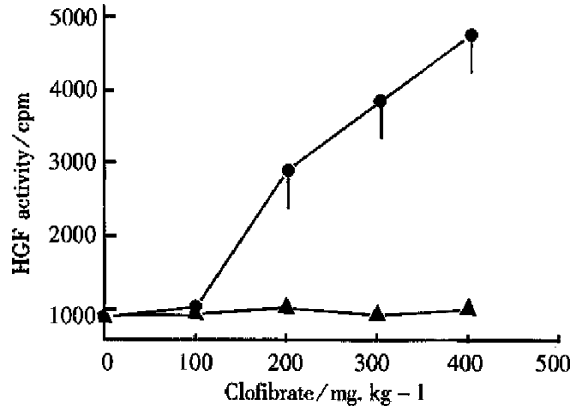

Figure1 Dose-effect curve of the effect of clofibr ate on the HGF level in rat serum 4 days after clofibrate administration.
▲-▲: Normal control group. ●-●: Clofibrate administration group.

As shown in Figure 1, in the normal control group, the cpm value caused by the incorporation of ^{3}H-TdR in the primary liver cells was 800±150, as it was promoted by the serum of the normal rats. However, in the clofibrate (100 mg·kg^{-1}·d^{-1}) group, the undiluted rat serum caused significant increase of the cpm value to 1010±180. Moreover, in pace with the increasing dosage of clofibrate, the cpm value increased also. When the dose of clofibrate increased to 500 mg·kg^{-1}·d^{-1}, the cpm value reached as much as 4800±810, so the dose-effect relationship was eminent.

Rats were given 500 mg·kg^{-1}·d^{-1} of clofibrate with their serum collected 4 days after. We could see that when the serum was diluted sequentially, the increased cpm value would fall down gradually, showing good dose-effect relationship. After the serum was diluted five-fold, the cpm value was still higher than that of the normal control group (Figure 2).

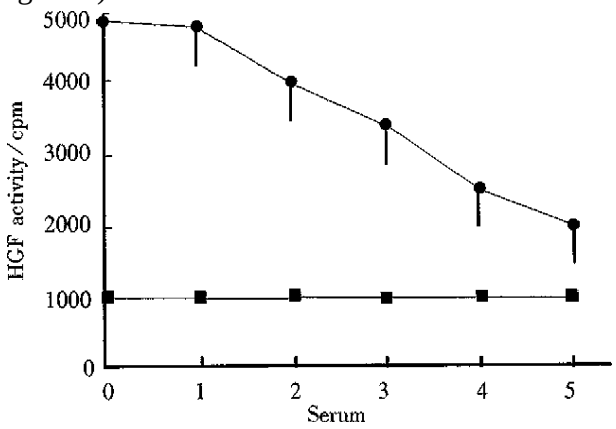

Figure 2 Variation of HGF level in rat serum 4 days after clofibrate administration.
■-■: Normal control group, ●-●: Clofibrate administration group.

Variation of the weight of rat liver and the HGF level in rat serum at different time intervals after clofibrate administration

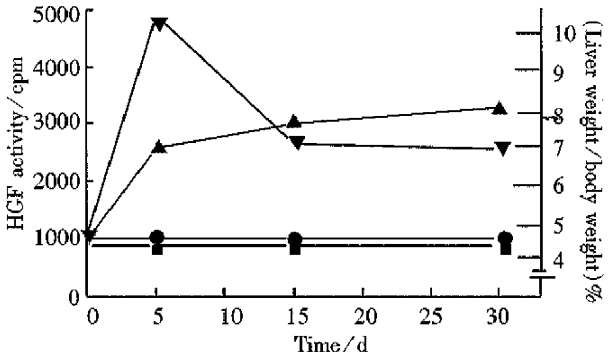

Figure 3 Variation of liver weight and serum HGF level in rats after clofibrate administration.
■-■: HGF level in normal control group,▼-▼: HGF level in clofibrate administration group, ●-●: Liver weight of normal control group, ▲-▲: Liver weight of clofibrate administration group.

As shown in Figure 3, 4 days after clofibrate administration, HGF level in serum reached its peak, and then fell gradually. Two weeks after taking clofibrate, HGF level still remained high, about 2-fold of the normal level while hepatomegaly also reached its highest level 2 weeks after intake of drug, and remained at this high level (1.8-fold above the normal level).

DISCUSSION

It was discovered in our study that after administration of clofibrate which can induce hepatomegaly, the HGF activity in rat serum increased significantly.

Previous studies by other investigators indicated that 24 h after carbon tetrachloride (a hepatotoxin) administration, the HGF level in rat plasma increase d by over 20-fold of the normal level[3]. In patients with partial hepa tectomy or with severe fulminating hepatitis, the releasing of HGF into plasma was closely related to liver injury[4]. Many experiments indicate that clofibr ate-related compounds can induce hepato megaly, proliferation of hepatic peroxisomes, and hepatic carcinoma[6]. It is shown in our experiments that the release of HGF induced by clofibrate rea ched a high level 4 days after the drug administration, and could maintain for a long time, while hepatomegaly reached its peak 2 weeks after drug administration and remained at a relatively steady level. So the HGF level is somewhat related to hepatomegaly, suggesting that hepatomegaly induced by clofibrate may depend partly upon the promoting action of HGF on liver regeneration. Although the mechanism of liver injury by clofibrate and carbon tetrachloride is different, both of them can induce liver injury, which is a signal leading to the generation of HGF. Via the action of HGF. DNA synthesis in liver is promoted, which ultimately results in repair and regeneration of the injured liver. Therefore, HGF plays an import ant role in the repair and regeneration of liver. However, the mechanism through which the release of HGF is promoted by these two compounds (clofibrate and carbon tetrachloride) is still unclear and needs further studies.

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The effect of retinoic acid on Ito cell proliferation and content of DNA and RNA

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Subject headings liver fibrosis; retinoic acid; Ito cell; cell culture; microspectrophotometer; DNA; RNA

INTRODUCTION

The development of liver fibrosis is due to an imbalance between synthesis and degradation of extracellular matrix. Recent studies have shown that Ito cells which are located along the sinuses of liver, can store and metabolize Vit A, and are the main cells that produce collagen, among which type I, III and IV and laminin, account for 80%-95% of the total hepatic collagen[1]. Ito cells in the course of proliferation and synthesis of collagen showed a reduction of Vit A contents and retinoic acid receptors[2]. This study was designed to investigate the effect of retinoic acid on Ito cell proliferation and the contents of DNA/RNA and to analyse further its mechanism or pathways.

MATERIALS AND METHODS

Ragents

DMEM medium (Gibco Compony); all trans-retinoic acid (RA), retinol palmitate (RP) (Sigma Company); ^{3}H-TdR (Shanghai Nuclear Energy Research Institute); Solution A: 0.1% Triton X-100, 0.08N HCl, 0.15N NaCl; solution B: 120μM acridine orange (AO), 1mM EDTA, 0.15N NaCl; vidas microspectr ophotometer analysis system.

^{3}H-TdR incorporation test

The isolation and culture of Ito cells were done by the method as published before[3]. Rat Ito cells of 5-day primary culture were suspended on DMEM me dium containing 20% bovine serum. The number of the cells was adjusted to 1×10^5/mL. One mL of the cell suspension was put into each well of the 24 -well culture plate, which contained serial concentrations of RA/RP. The control wells contained no RA/RP. After incubating for 24 h, 8 μci-^{3}H-TdR was added to each well and incubated for another 48h. The cells were collected on the F49 filter paper, fixed with 4mL 10% trichloro-acetic acid and dehydrated with 4mL alcohol. The dried filter papers were put on the bottom of the scinti bottle which contained 5 mL scintillating solution for counting the pulse per minute (CPM). The degree of DNA replication was indicated by CPM/well. We set three wells for each sample.

DNA/RNA content analysis

Ito cell culture and treatment were handled with the same procedure as above and the difference was as follows: drug treatment with RA/RP at 10^{-4}mol/L, small cover glass was put into each well of the plate to bear the growing cells. After the culture medium was discarded, the Ito cells on the small cover g lass were fixed for 30min with 70% alcohol and washed with PBS solution. Solution A (0.4mL) was added into each well, the preparation was set onice bath for reacting 15 seconds, 1.2mL solution B was then used for 8min staining. The small cover glass was put on a glass slide, fluorescent microscopic examinati on was made immediately with the inciting light wave of 488 nm. DNA was examined with a screened filter at a wave-length of 530 nm, with green in stain, whereas RNA examined with a filter at 610 nm showed a red fluorescence. Fifty cells were examined in each group with fluorescent microspectrophotometer-30 (FMSP-30); the fluorescence intensity was converted into grey scale value, corresponding to the relative aver age DNA/RNA contents of Ito cells.

RESULTS

^{3}H-TdR incorporation test

Lower densities of RA/RP (10^{-6} mol/L) had no effect on the ^{3}H-TdR incorporation of Ito cell. With higher densities ^{3}H-TdR incorporation of Ito cell was inhibited as compared with the control (P<0.05)(Table 1).

DNA/RNA content analysis

The results showed that RA/RP at 10^{-4}mol/L

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could reduce DNA/RN A- contents of Ito cells as compared control (P<0.01) (Table 2).

Table 1 ^{3}H-TdR incorporation test

Group	Dosage (mol/L)	(CPM±s)/well
Control		2866.4±253.2
Test groups		
RA	10^{-6}	2657.6±104.8
	10^{-5}	1279.4±236.4[a]
	10^{-4}	182.2±67.6[a]
RP	10^{-6}	2598.4±76.4
	10^{-5}	1182.6±154.4[a]
	10^{-4}	897.2±82.6[a]

[a]P<0.05 *vs* control group (Student's *t* test).

Table 2 DNA, RNA content analysis

Group	Dosage (mol/L)	DNA	RNA
Control		101.98±21.58	89.38±22.03
RA	10^{-4}	61.79±18.31[a]	56.31±14.72[a]
RP	10^{-4}	46.85±11.52[a]	49.20±10.12[a]

[a]P<0.05 *vs* control group (Student's *t* test).

DISCUSSION

Ito cells which have the characteristics of fibroblasts cell and myofibroblasts are the main collagen-producing cells in the liver. Bamard H[4] and Seifert WF[5] reported that RA could reduce the deposition of types I and III collagen in the CCl_4-induced liver fibrosis of the rat through its inhibitory effect on the transformation of Ito cell to myofibroblasts.

Our study showed that RA/RP could inhibit ^{3}H-TdR incorporation of rat Ito cells and reduced the DNA/RNA contents of rat Ito cells. Our previous study indicated that RA could restore retinoic acid receptor content and increase cAMP content in the primary culture of rat Ito cells[6]. The form of biologic effect of RA was similar to that of thyroxin[7], i.e. they both act on nuclear receptor resulting in a change of the second messenger and regulating the gene expression of RAR and collagen in the Ito cells. RA may be expected to be an effective antihepatofibrotic agent.

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TThe mutation induced by space conditions in *Escherichia coli*

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Subject headings Escherichia coli; mutation; space conditi ons; microorganism breeding

INTRODUCTION

Progress has been made in microorganism breeding under space conditions by board ing on recoverable satellite and high altitude balloon in China. To further study the mutagenesis in space, three strains of *E. coli* were put on board the recoverable satellite (JB1-B9611020) launched in October. 1996. After the satellite returned to the earth, the survival and mutation frequencies were determined and the results were discussed as well.

MATERIALS AND METHODS

Bacterial strains

CSH108[1], an arginine autotrophic (Arg) strain, was provided by the *E. coli* Genetic Stock Center, USA (CGSC) and was used to study Arg^+ reversion mutation. Both Arg^- and $LacZ^-$ in CSH108 were caused by amber mutation. The strain A2 and A3 were constructed for $lacI^-$ mutation in this study (the detailed procedure was not described here). A3 was a lacI-qstrain used for the selection of $LacI^-$ mutation after boarding, while A2 is a $lacI^-$ strain serving as a control strain. The properties of flight *E. coli* strains are listed in Table 1.

Table 1 Properties of the boarded E. coli strains

Strain	Genotype
A2	*ara(lac proB)strA/F'lacI* $^-$ *proA*$^+$*B*$^+$
A3	*ara(lac proB)strA/F'lacI*^{q} *pro*$^+$
CSH108	*ara(gpt-lac)gyrA argE*$_{am}$ *proB/F'lacI*$^-$ *Z*$^-$*proA* $^+$*B*$^+$

Boarding methods and space conditions

Since *E. coli* strains usually are hard to survive from space board, soft agar culture of *E. coli* cells was made in this boarding. It was prepared as below: the cells grown on plate were suspended in a small amount of LB liquid medium, and then added melted sterile agar to get soft agar culture (the final concentration of agar was 3.5 μg/L).

In order to study the mutagenesis induced by different factors in space conditions, the boarding samples were divided into three groups and each group included the three strains. When boarding on the satellite Group I was held in small polymethyl methacrylate tubes, Group II was placed in a centrifuge inside DM-11 small biocabin, where oxygen was supplied[2] and the gravity was adjusted to 1g, and Group III was placed in a lead chamber (usually used to store radioisotope) which had 3.5 mm-8.0 mm thick wall and coated with 5 mm layer of hard plastics outside. The lead chamber could block part of t he radiation, but the exact amount of block efficiency was not determined in this study. Part of the ground control bacteria was placed in a dark vessel at room temperature (13 ℃-20 ℃), the other was stored in a freezer (-60 ℃).

The satellite flew for 15 days. The angle of satellite orbit was 63°, apogee was 354 km, perigee was 175km, microgravity was 5×10^{-5} g, density of high energy particle was 136 counts/cm^2 [it was (35.6±6) counts/cm^2 on the earth]. The records show that the biocabin worked regularly in flight, in which the temperature was 17 ℃-26 ℃ and the mean dosage of ionizing radiation was 0.177 mGy/d.

Mutation frequency of the bacteria

The mutation frequency was measured soon after the flight. The procedure was as follows: 0.5 mL of the boarded and control samples were inoculated in 5 mL of LB medium respectively. After 4 h at 37 ℃, 2.5 mL of 50% sterile glycerol was added, and all samples were divided into 1 mL of aliquot, and stored at -20 ℃. During the measurement, an aliquot of the sample was used for bacterial cell counting, and then concentrated sample was spread on screening plates to select mutants. The number of mutants was scored in 48 h-72 h incubation at 37 ℃. The mutation frequency of bacteria was calculated according to the formula: The mutation frequency=mutant cells per mL/total bacterial cells per mL.

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Media

The LB medium, minimal medium, MacConkey medium and Pgal medium were used in experiment as described[1].

Selection of Arg^+ reversion mutants and test of Arg^+ Lac^+ mutants

The concentrated CSH108 sample was spread on minimal plate without arginine. The Arg^+ revertants could grow after incubation. Then the Arg^+ revertants were streaked on minimal plate with lactose as the only carbon source. If some of the revertants could grow up on the medium, they were Arg^+ Lac^+ revertants. These were determined by the method of Miller[1].

Selection of $LacI^-$ mutant

Phenyl-β-D-galactoside (Pgal) is a noninducing sugar that se rves as a substrate for β-galactosidase and can provide a carb on source for growth, but only the constitutive mutants of $LacI^-$, which produce enough β-galactosidase, can form colonies on Pgal[1]. To prove to be $LacI^-$ mutants, the colonies grown on the Pgal plate were streaked on MacConkey plate to compare with A2 strain as the $LacI^-$ control.

RESULTS

The survival of the boarded strains

Before boarding, the cell counting of samples was up to 2×10^9/mL and after flight the survival of each sample was about 3×10^8/mL counted on LB plate, suggesting that the survival of the strains was accomplished as expected in this study.

The Arg^+ reversion frequency of strain CSH 108

After flight the Arg^+ reversion frequencies of CSH108 in the three groups and the ground control were determined. The results are shown in Table 2 (the reversion frequency of each was the mean of seven tests). In fact the Arg^+ reversion frequency of the ground control was the spontaneous reversion frequency. It was worth mentioning that the Arg^+ reversion frequency of Group III was 10 times that of the ground control.

Table 2 The Arg^+ reversion frequency of strain CSH108 in different boarding ways

Sample	Group	Arg^+ reversion frequency($\times10^{-8}$)
Boarding sample	Group I	2.9
	Group II	1.8
	Group III	26.3
Ground control	A(1)	1.2
	B(2)	2.8

(1) Ground control: strain was kept at room temperature; (2) Ground control: strain was stored in freezer (-60℃).

The Lac mutation frequency among Arg^+ revertant

The Lac^+ mutation frequency of CSH108 varied with the groups. As shown in Tables 2 and 3, Group III had not only a high Arg^+ reversion frequency, but also a high Arg^+ Lac^+ frequency. It is suggested that most of the Arg^+ revertan ts were suppresser mutations, which resulted from mutations located in tRNA genes.

Table 3 The occurrence of Lac^+ phenotype among Arg^+ revertants

Sample	Group	Total No. of Arg^+ reversion	No. of Lac^+	Lac^+/Arg^+ (%)
Boarding sample	Group I	60	25	41.7
	Group II	139	52	37.4
	Group III	384	376	97.9
Ground control	A(1)	36	14	38.9
	B(2)	39	27	69.2

A(1) and B(2) are the same with that in Table 2.

The $LacI^-$ mutation in A3 strain

In A3 strain, the survival and $LacI^-$ frequencies are shown in Table 4. The $LacI^-$ frequency in Group II was remarkably higher than that in other groups and it was 67 times that of the ground control. In addition, we also observed that when the boarded strains were plated on Pgalagar at 37℃, Group II formed colonies in 48h, while other groups formed colonies in 72h. From Table 4, it also can be seen that the $LacI^-$ mutation frequency in Group III was 4.4 times that in the ground control. A further test showed that most of the $LacI^-$ mutation in A3 strain could not be suppressed in suppresser strains, therefore they were not amber mutations (The detailed result was not described here).

Table 4 The survival and frequency of $LacI^-$ mutant from A3 strain

Sample	Group	Survival ($\times10^8$)	$LacI^-$ frequency ($\times10^{-8}$)
Boarding sample	Group I	3.6	0.4
	Group II	5.5	240.0
	Group III	3.0	15.8
Ground control	A(1)		3.6

All figures were means of four tests. (1) Ground control: strain was kept at room temperature.

DISCUSSION

Arg^+ revertant

Reversion mutation is a simple and accurate method used to determine the mutation frequency of bacteria[5]. At least two kinds of mutations can reverse the Arg^- (arginine synthesis defective)

phenotype in CSH108: the mutation at $ArgE_{am}$ position and the suppresser mutation. They are both point mutations, but occur in different places. In the revertants with only Arg^+ phenotype, the mutation results from a base substitution at $ArgE_{am}$ position to restore Arg^+ by a sense triplet; in the revertants with Arg^+ Lac^+ phenotype, the mutation occurs in tRNA gene and gets the intergenic repressor by suppresser mutation[1,3]. According to the results shown in Table 3, the revertants of suppreser mutation in Group III covered 97%, while the frequen cies of such mutation were below 70% in other groups, usually about 50% (Table 3).

LacI⁻ mutant

lacI gene encoded repressor for *lacZ* gene. $LacI^+$ bacteria could not grow in Pgal plate unless they were mutated to $LacI^-$ strain. According to the results in Table 4, the $LacI^-$ mutation frequency in Group II was 67 times that of the ground control, and was 4.4 times in Group III that in the ground control. Both mutation and reversion are often used in microorganism genetic experiment[1]. It is convincing to use the markers in this study to investigate the mutagenesis of microorganism in space conditions.

Boarding methods

Three boarding methods were used, and the reversion mutation frequency of Arg^+ and the mutation frequency of $LacI^-$ were measured in this study. The samples of Group II were placed in DM-11 small biocabin, therefore the microgravity had little effect. The main factor affecting the samples was space radiation. In addition, oxygen was supplied in biocabin. It had been reported that in mammalian cell the break incidence of single-stranded DNA in O_2 environment was four times higher than that in no O_2 environment[4], and the occurrence of mutation was closely related with the repair of DNA damage[3,5]. These are probably the reasons why the $LacI^-$ mutation frequency of A3 strain in biocabin was 67 times that of the ground control.

The small lead chamber could block part of the space radiation in flight. The results showed that in lead chamber the Arg^+ reversion frequency of CSH108 was 10 times that of the ground control, and that the $LacI^-$ mutation frequency of A3 strain was 4 times that of the ground control. In this test, the effect of space radiation was decreased by the chamber, the main effective factor should be the microgravity.

The boarded Group I was influenced by microgravity and strong space radiation, but no significant effect on mutation in *E. coli* strains was found. It may be due the interference of the samples located in the satellite, or the antagonis m between different space factors, or some unknown reasons.

It was shown that after flight some *E. coli* strains had high mutation frequencies which varied with boarding conditions. That is to say, in different boarding conditions, there were different space factors that influenced the bacteria mutagenesis, therefore different types and frequencies of mutations were induced. The effects of spaceflight have been increasingly understood[1]. This research indicated that the spaceflight may greatly enhance the mutation frequency of certain genes in microorganism and may provide an effective way for microorganism breeding. But space factors, such as strong radiation, microgravity and so on, which influence the mutation of *E. coli* are complicated. The mutation effect would vary with strain, gene, and even the nucleotide location in DNA. Therefore much work is to be done in understanding the mechanism of space induced breeding. In addition, it is meaningful to take the advantages of quick growth and clear selective markers in *E. coli* strains to develop a high-speed routine method for predicting the space induced efficiency. The method can serve all the purposes of biological investigation.

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Treatment of corticosteroid-resistant ulcerative colitis with oral low molecular weight heparin

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Subject headings colitis; ulcerative/drug therapy; heparin/therapertic use; corticosteroid-resistant

INTRODUCTION

The etiology and pathogenesis of ulcerative colitis (UC) have remained unclear. Treatment is nonspecific based on the anti- inflammatory agents corticosteroid and sulfasalazine. A significant proportion fail to respond to this therapy[1]. As the relapse, refractory or serious UC patients had a hypercoagulable state and an increased incidence of thromboembolic events[2-4], heparin has been used by some authors[5-7]. Yet, its half-life period is short, needing long-term injection, which restricts its further clinical application. Our previous studies have demonstrated oral LMWH not only overcomes the shortcomings of common heparin[8,9], but also has anti-inflammatory effects[10,11]. The aim of this paper is to study the therapeutic effects and mechani sm of oral LMWH in patients with corticosteroid-resistant UC.

MATERIALS AND METHODS

Clinical materials

There were eight men and twelve women aged 21 years to 56 years (mean 33 years). All cases were histologically confirmed and met the diagnostic standard of chronic non-infectious intestinal disease of China (Taiyuan meeting, 1993), including seventeen cases of severe, and three moderate UC. Duration of diseases ranged from 8 months to 11 years (mean 4.1 years). Rectal bleeding, diarrhea, mucus stool, abdominal pain were the main symptoms. Four patients were associated with thromboembolic diseases. All patients were treated with high-dose corticosteroi d and sulfasalazine for more than 4 weeks without effect, sulfasalazine was maitained in combination with oral LMWH (366U/kg, twice daily) for more than 4 weeks. Prednisolone was tapered and stopped.

Monitoring parameters

Assessment of platelet activation and aggregability[2,4]. We used a sensitive flow cytometric technic designed to minimize sample handling and render fixation unnecessary to quantify platelet activation. Blood samples were incubated by 10 minutes of venesection with fluorescein isothiocyanate (FITC) conjugated antibodies to the platelet surface antigens, P-selectin (CD_{62P}) and CD_{63} (Immunotech, Marseilles, France). Analysis was made within 15 minutes of venesection using a BD (Becton Dickinson Immonocytometry Systems) FAC Scan. TXA-2 (Suzhou Medical College) was measured using RIA method, samples wer e taken without tourniquet into chilled tubes containing 1:9 anticoagulant/antiaggregant solution (trisodium cirrate 3.8%), centrifuged for 15min-30min, later at 4 ℃ for 30 minutes to minimize *in vitro* activation, supernatant was decanted off and stored at -20 ℃ for assay within 3 months. Platelet aggregation rates (PAR) and thrombosis length (TL) *in vitro* were assessed by XSN-R II instrument according to the manufecturer's instruction.

Measurment of CD_{54}. CD_{54} in blood and tissues were measured using flow cytometric technic according to our previous report[12].

Assessment of efficacy[7]

Pre- and post-treatment scores were calculated for the following disease parameters: ① Stool frequency (average number per day for the past week). ② Rectal bleeding (0: absent, 1: streak of blood on stools occationally, 2: obvious blood on stool frequently, 3: complete bloody stools). ③ Colonoscopic appearance 0: normal vascular pattern, 1: mild lesion (loss of vascular pattern, mucosa edema, no bleeding), 2: moderate lesion (granularity and friability of the mucosa), 3: severe lesion (discrete ulceration and spontancous bleeding). ④ Histological grading: serial biopsies of the rectum and the colon were taken. Five histological chang es seen in UC (cellular infiltrate in the lamina propria, cryptitis, crypt abscess

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formation, golet cell depletion, and regenerative hyperplasia of the epitheli um) each were scored from 0 (absent) to 3 (severe), a total UC score of 5 or less indicated mild disease, a score of 5-10, moderate, and a score of 10-15 severe desease. ⑤ General health status (0: excellent, 1: good, 2: poor, 3: poorer, 4: very poor, 5: poorest).

Statistical analysis

Student's *t* test and Friedman test were used to assess the significance of differences between mean pre- and post-treatment parameters.

RESULTS

Therapeutic effects

Nineteen patients (95.0%) achieved clinical remission (normal stool frequency and no rectal bleeding) on a combination of oral LMWH and sulfasalazine. One patient had reduced rectal bleeding only. The average period of marked improvement was 2.9 weeks (range 1 week-4 weeks), and of remission was 5 weeks (range 1 week-12 weeks). Rectal bleeding ceased in 19 patients (5 patients within 5 days -8 days, the others within 2 weeks-7 weeks). Nineteen patients had general health condition improved earlier on oral LMWH, than bowel symptoms. There were highly significant improvement in mean scores for all disease parameters (Table 1).

Table 1 Therapeutic effects of oral LMWH in corticosteroid-resist ant UC patients

Group	Stool frequency (times/day)	Rectal bleeding (score)	Colonoscopy (score)	Histology (score)	Well-being (score)
Pre-treatment	8.6	2.6	2.7	12.0	4.0
Post-treatment	1.5[b]	0.2[b]	1.0[b]	4.0[b]	0.6[b]

[b]P<0.01 *vs* pretr eatment.

Blood contents of CD_{62P}, CD_{63}, TXA^2, platelet aggregation rate (PAR) and thrombosis length (TL) in vitro

All the indexes in corticosteroid-resistant UC patients increased significantly as compared with the normal controls (P<0.01). After treatment with oral LMWH, all the parameters of UC patients decreased (P<0.01), but CD_{62P} and CD_{63} remained higher than normal (P<0.01), (Table 2).

Table 2 Effects of oral LMWH on CD_{62P} and CD_{63}, TXA_2, platelet aggregation rate (PAR) and thrombosis length (TL) *in vitro* in UC patients ($\bar{x}$±s)

Group	CD_{62P}(%)	CD_{63}(%)	TXA_2(ng/L)	PAR(%)	TL(cm)
UC patients					
Pre-treatment	8.1±3.2[b]	6.2±2.2[b]	541.7±82.4[b]	44.5±10.1[b]	2.4±0.5[b]
Post-treatment	4.2±1.9[a,d]	3.1±1.7[ab]	396.4±75.8[d]	35.2±8.7[d]	1.9±0.4[d]
Normal controls	1.9±00.4	1.6±0.8	340.2±40.4	34.1±9.1	

[a]P<0.05, [b]P<0.01 *vs* normal person; [d]P<0.01 *vs* pretreatment.

CD_{54} in blood and tissues

CD_{54} elevated in both blood and tissues in corticosteroid-resistant UC patients ($P < 0.01$), CD_{54} in tissues being higher than in blood. After oral LMWH, CD_{54} lowered significantly in both blood and tissues ($P < 0.01$), but still higher than that of normal controls (P<0.05), (Table 3).

Table 3 Effects of oral LMWH on CD_{54} in UC patients

($\bar{x}$±s,%)

Group	Blood CD_{54}	Tissue CD_{54}
UC patients		
Pre-treatment	28.7±6.1[b]	50.7±6.8[b]
Post-treatment	14.6±5.2[a,d]	22.8±4.7[a,d]
Normal controls	6.2±3.7	8.8±3.2

[a]P<0.05, [b]P<0.01 *vs* normal; [d]P<0.01 *vs* post-treatment.

Complications

No serious complications were associated with the use of oral LMWH.

DISCUSSION

Heparin, a group of sulphated glycosaminoglycans, in addition to its physiological effects and anticoagulant, antithromboembolic, antiallergic, antiviral, antiendotoxic and immunoregulative biological activities, has a wide range of potentially anti-inflammatory effects, including inhibition of neutrophil elastase and inactivation of chemokines[5,13]. Compared with heparin, LMWH has a enhanced antithromboembolic effects, longer half life period, less bleeding tendency, higher bioavailability, easier absorption by oral administration[8,9], and has the anti inflammatory effects as well[10,11]. Previous reports[5-7] on improvement in UC patients treated with heparin prompted us to perform a pilot study of oral LMWH to find a more convenient and effective drug for patients with corticosteroid-resistant UC. The observed response to oral LMWH is paradoxical. Nineteen of 20 patients with corticosteroid-resistant UC achieved clinical remission and became asymptomatic on oral LMWH combined with sulfas alazine. Opposite to the traditional idea that heparin can enhance bleeding, rectal bleeding was the first symptom to be improved by oral LMWH. The results are similar to other reports of heparin treatment[5-7].

If oral LMWH has a therapeutic effect in UC, its mechanism of action should shed some light on the elusive pathogenesis of this disease. There are several thrombophilic features of UC that suggest the effect of oral LMWH on colitic symptoms may be attributable to its anticoagulant and antithrombotic properties. Evidence of a

thrombotic process in UC includes: reports of a hypercoagulable state[2-4], an increased incidence of thromboembolic event[14], and ischemic complications such as toxic megacolon and pyoderma gangrenosum. In this study, the membrane marks of platelet activity CD_{62P} and CD_{63} increased significantly, and the derivative of active platelet TXA-2 also elevated, sugge sting that the blood platelet was in an active state, which not only led to a hy percoagulable state and an increased incidence of thromboembolic events, but also enhanced inflammatory reaction[24]. Activated hyperaggregable platelets in the mesenteric circulation could amplify the inflammatory cascade by promoting neutrophil recruitment and chemotaxis. P-selectin has an established action as the adhesion molecule for neutrophils, and circulating platelet aggregates may contribute to ischemic damage and infarction by occluding the intestinal microvasculature. Platelet derived thromboxane A_2 may also contribute to the ischemia by inducing local vasocontriction. After treatment with oral LMWH, all these par ameters dropped markedly, suggesting that the therapeutic effect of LMWH is partly related to inhibition of platelet activity[9]. CD_{54} antigen reacts with the 85 kD-110 kD integral membrane glycoprotein, is also known as an intercellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells and both resting (weak) and activated (moderate) lymphocytes and monocytes. CD_{54} is ligand for the leukocyte function antigen-1 (CD_{11a}. Its expression is up- regulated upon stimulation by inflammatory mediators such as cytokines and LPS, and it is involved in B cell-T cell co-stimulatory interations. In this study, CD_{54} elevated significantly in blood and tissues of UC patients, being in tissues higher than in blood[12]. Therefore, it could reflect the inflammation of intestinal mucosa. After oral LMWH, CD_{54} dropped significantly in both blood and tissues, indicating that oral LMWH could relieve the inflammat ory activity in these patients who received prednisolone for a long period (more than 4 weeks) and had no significant improvement and were regarded as corticost eroid-resistant refractory cases of UC. In other reports[5], heparin can also inhibit c-reactive protein (CRP), tumor necrosis factor (TNF) and L-se lectin of UC patients. The detailed mechanisms by which the anti-inflammatory properties of oral L MWH are mediated in UC remain to be elucidated further.

From these results, we conclude that oral LMWH may play a role in treating corticosteroid-resistant UC, the mechanism is partly related to inhibition of platelet activity, hypercoagulable state and anti-inflammatory effects. No serious complications were found associated with the use of oral LMWH.

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Methylation status of *p16* gene in colorectal carcinoma and normal colonic mucosa

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Subject headings Colonic mucosa; colorectal neoplasms; *p16* gene; methylation

INTRODUCTION

p16 gene (also known as MTS-1, INK4a, CDKN2A), located on chromosome 9p21, is a G1-specific cell-cycle regulatory gene. It is composed of three exons, which encode 156 amino acids[1]. The gene is frequently inactivated in many human cancers. Unlike other tumor-suppressor genes that are commonly inactivated by point mutations, small homozygous deletions and methylation of the promoter represent the major mechanism of *p16* gene inactivation[2,3]. In the Western countries, colorectal carcinoma ranks first among malignant tumors. The mortality from colorectal carcinoma has been rapidly growing in China in the last two to three decades. The genetic alterations involved in this tumor are still unclear. Rare mutation and infrequent deletion of *p16* gene in primary colorectal car cinoma has been widely reported[4]. There were a few papers on *p16* gene methylation in colorectal carcinoma, but the results were contradictory[5,6]. In this study, we have examined a total of 60 samples of colorectal carcinoma and paired 60 samples of the normal colonic mucosa for methylation by means of PCR-based methylation assay. There is infrequent methylation in the promoter of *p16* gene both in colorectal carcinoma and normal colonic mucosa. The methylation status in 5' CpG island of *p16* gene in colorectal carcinoma is not re lated to the clinical pathologic parameters of these tumors.

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MATERIALS AND METHODS

Patients and specimens

The samples were provided by the First and Second Affiliated Hospitals of Zhejiang Medical University and Hangzhou Railway Central Hospital from 1996 to 1998. C olorectal carcinoma specimens ($n = 60$) and matched normal colorectal mucosa ($n = 60$) were obtained from 60 patients (38 men and 22 women) with colorectal carcinoma. Their ages ranged from 20 to 78 years with a mean age of 57. The size of the tumors ranged from 1.5 cm to 7.8cm in diameter.

Template preparation

DNA was isolated by proteinase K digestion and phenol chloroform extraction. The DNA concentration and purity were determined on the ultraviolet ray spectrometer (Pharmacia Bioth Ultrospec 2000). All DNA templates were diluted to 1 μg/μL with TE.

PCR-based methylation assay

Genomic DNA (1 μg) was either overdigested with 20 U three methylation-sensititive restriction enzymes (*Hpa* II, *Sac* II and *Sma* I) or placed in the appropriate buffer without enzyme (control) overnight. A liquots of 120 ng of the digested and non- digested DNA were PCR amplified using primers 5'GAAGAAAGAGGAGGGGGTG-3' (sense) and 5'GCGCTACCTGATTCCAATTC-3' (antisense), which corresponded to a part of 5' noncoding region and a part of the 3' end of exon 1 of the *p16* gene. The undigested DNA of the correspondent samples was also amplified and used as control. A total of 25 μL volume of PCR mixture contained 2 μL of 10×buffer, 0.8μL of $MgCl_2$ (25 mM), 2 μL of dNTP (0.2 mM), 2 μL of primer (20 pM), 15 U of Taq polymerase and 2.5 μL of digested DNA. Amplifications were performed in a temperature cycler (MJ Research, Inc., USA) for 1 cycle at 95 ℃ (5 min), 30 cycles at 95 ℃ (30 s), 55℃ (30 s) and 72 ℃ (30 s), and 1 cycle at 72 ℃ (5 min). After PCR, the reaction mixture was electrophoresed in 1.5% agarose gel and examined to find out whether a 340 bp long product was generated. The sample which is unmethylated and cut by restriction enzyme, has no amplified product while the sample which is methylated and not cut by

restriction enzyme, has amplified product.

Statistical analysis

Statistical analysis was made by Chi-square test. The difference was regarded significant when *P* value is less than 0.05.

RESULTS

Methylation of the *p16* gene in colorectal carcinoma and normal colonic mucosa. We have investigated the biopsies of 60 cases of primary colorectal carcinoma and paired 60 cases of normal colonic mucosa for methylation of the *p16* gene in *Hpa*-II, *Sac*-II and *Sma*-I sites. All of the undigested control of 60 cases of colorectal carcinoma and 60 cases with normal colonic mucosagot the same PCR products of 340 bp, while the digested samples achieved the different results (Figure 1). In the normal colonic mucosa, at the Hpa-II site in 5'CpG island of *p16* gene, 13 (21.6%) of 60 samples showed full methylation and 7 (11.6%) samples partial methylation, at the *Sac*-II site 28 (46.6%) of 60 showed full methylation and 8 (13.3%) partial methylation and at the *Sma*-I site, 5 (8.3%) showed full methylation and 4 (6.7%) partial methylation. In the colorectal carcinoma, at the *Hpa*-II site, 11 (18.3%) samples showed full methylation and 10 (16.7%) partial methylation, at the Sac-II site, 22 (33.3 %) full methylation and 6 (10%) partial methylation, and at the *Sma*-I site, 9 (15%) full methylation and 3 (5%) partial methylation. In comparison of the methylation status of colorectal carcinoma and that of normal colonic mucosa, there was no significant difference ($P>0.05$).

Statistical analysis revealed a marked significant difference in the methylation status of *Hpa*-II, *Sac*-II and *Sma*-I sites of *p16* gene in normal colonic mucosa ($\chi^2 = 28.6, P < 0.01$), and the methylation of pro moter in *p16* gene tended to be associated with aging ($\chi^2 = 5.64$, $0.1>P>0.05$) (Table 1).

Table 1 Correlation between methylation of *p16* gene and age, enz yme cutting site of normal clonic mucosa

	Number of site	Full methylation	Partial methylation	Un-methylation
Age (yrs)				
≤40	33	4	3	26[a]
41-60	60	19	8	33
>60	87	23	8	56
Site				
Hpa-II	60	13	7	40[b]
Sac-II	60	28	8	24
Sma-I	60	5	4	51

[a]$0.1>P>0.05$, [b]$P<0.005$.

The 60 cases of colorectal carcinoma were divided into groups according to the pathological types and size of cancer and the lymph node metastasis. It was found that the methylation of 5' CpG island of the *p16* gene was not related to the clinicopathologic parameters (Table 2).

Table 2 Correlation between methylation of *p16* gene and clinicop athologic parameters of colorectal carcinoma

	Numbers of site	Full methylation	Partial methylation	Un-methylation
Histological type (adenocacinoma)				
Well-differentiated	18	6	3	9[a]
Moderately-differentiated	117	26	14	77
Poorly-differentiated	15	3	2	10
Mucinous	21	6	0	15
Size (cm)				
≤2.5	27	5	4	18[a]
2.6-4	54	15	5	34
>4	87	21	10	56
Lymph node metastases				
With LN metastases	78	20	5	53[a]
Without LN metastases	102	26	14	62

[a]$P>0.05$.

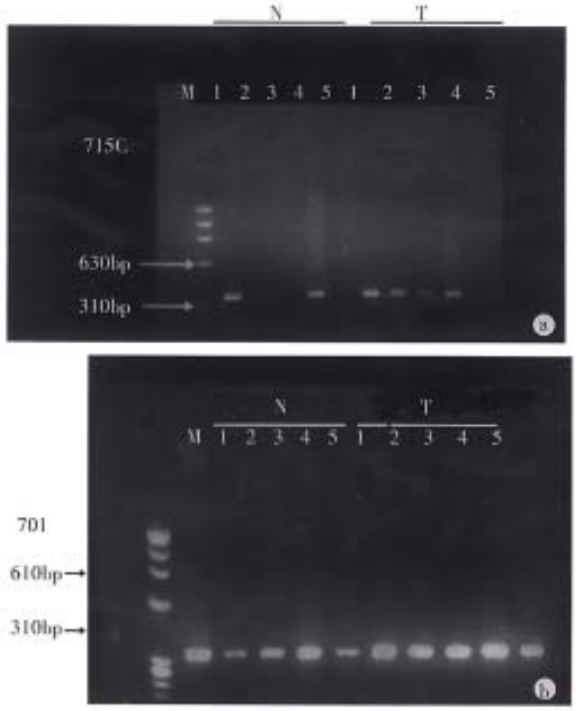

Figure1 PCR-based detection of 5' CpG island meth ylation. N: normal colonic mucosa; T: colorectal carcinoma. A 5' CpG island sequence (34 0bp) of the *p16* gene was PCR amplified after sufficient digestion with a met hylation sensitive enzyme *Hpa*-II (2), *Sac*-II(3) and *Sma*-I(5) and undige sted control (1, 4). A: case 715c, in the normal colonic mucosa, *Hpa*-II and *Sma*-I sites unmethylation (N2, N5) and Sac-II sites partial methylation (N3); in the colorectal carcinoma, *Hpa*-II and *Sac*-II site partial methylati on (T2, T3) and *Sma*-I site unmethylation (T5). B: case 701, in the normal co lonic mucosa, *Sac*-II site full methylation (N3) and *Sma*-I and *Hpa*-II sites partial methylation (N5, N2); in the colorectal carcinoma, *Hpa*-II, *Sac*-II and *Sma*-I sites full methylation (T2, T3, T5).

DISCUSSION

There are two major mechanisms of gene inactivation. One is the genetic mechanism, i.e. the aberration of DNA structure such as homozygous deletion or intragenic mutation resulting in the gene inactivation. The other is the epigenetic mechanism, i.e., the methylation of the position 5 of cytosine (C) leading to the lack of gene expression, while the structure and the product of the gene remained unchanged. In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas kno wn as CpG islands, located in the promoter region of many genes. While almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes and the genes on inactivated X-chromosome of females. Aberrant methylation of normally unmethylated CpG islands has been associated with transcription inactivation of gene[7].

The exon 1 coding sequences of the *p16* gene resides within 5' CpG islands. This area is not methylated in most normal tissues but methylated in many human cancers. The rates of homozygous deletion and intragenic mutation of *p16* gene in colorectal carcinoma are very low, but how about the methylation of 5' CpG islands of *p16* gene in this tumor? We examined the methylation status of 3 40bp sequence within 5' CpG islands of *p16* gene in colorectal carcinoma and normal colonic mucosa using the PCR-based methylation assay. Our data suggest that there was no significant difference in the methylation status of *p16* gene 5' CpG islands between the colorectal carcinoma and normal colonic mucosa. We further studied the correlation between methylation of *p16* gene and clinicopathologic parameters of colorectal carcinoma, which also showed that the methylation of *p16* gene did not play an important role in the progress of colorectal carcinoma. Recently, some scholars suggest that the cell proliferation in colorectal carcinoma, unlike in most other tumor types, could bypass *p16*ink4a-mediated growth arrest[8]. Our experiment, which combined with the reports of low rates of homozygous d eletion and intragenic mutation of *p16* gene in colorectal carcinoma, support s this opinion.

There are contradictory results on the methylation of the 5' CpG island of *p16* in colorectal carcinoma and normal colonic mucosa reported by Herman *et al* and Gonzalez-Zulueta *et al*[5,6]. We found the methylation of the 5'CpG island of *p16* gene in colonic mucosa was very complicated. The full methylation rates of *Hpa*-II and *Sma*-I sites in 5' CpG island of *p16* gene were low (21.6% and 8.3%), while that of *Sac*-II site was high (46.6%). There were significant differences in methylation status of three different sites ($P<0.01$. It suggested that using different methylation-sensititive restriction enzymes in different experiments without self control might lead to contradictory results.

The existence of partial methylation status has also been verified. We could see the weak bands (1/ 2-1/8 of control) after the samples were digested by methylation-sensititive restriction enzymes in some cases. It was confirmed by repeated digestion. This could be explained by the presence of distinct cell subpopulati on. The other explanation is the methylation of one allele. As PCR-based technique facilitates the detection of low numbers of methylated alleles, the DNA of stroma may influence the results.

DNA methylation plays an important role in the development, imprinting and aging. It has been reported that the methylation of estrogen receptor gene CpG island links with aging in human colon[9]. In this experiment, the rates of the methylation of promoter in *p16* gene increased with aging in colonic mucosa.

Southern hybridization is a classical method for detecting methylation status of specific sequence in specific genes, it needs not only more DNA but also radioactive element. PCR-based methylation assay has provided significant advantages of being markedly more sensitive and highly efficient, and need no radioactive element and expensive instruments.

To avoid false positive results, the key problem of this method is the sufficient digestion of DNA templates with restriction enzymes. In this experiment, we diluted all the DNA sample to 1μg/μL, and selected the smallest numbers of template which could find clear PCR product by ladder diluted met hod. We digested the DNA with the largest amount of enzyme, prolonged the digestion time (overnight) and repeated digestion to some partial methylation cases to confirm results. We used the digested products without ethanol precipitation as template of PCR to avoid the loss of DNA and adjusted the reaction mixture in accordance with different buffers (*Hpa*-II, *Sac*-II with buffer A, *Sma*-I with buffer J) used in different restriction enzyme digestion. For each case, we amplified the digested template and the undigested one simultanously with the same

digestion buffers as control.

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Study on the expression of matrix metalloproteinase-2 mRNA in human gastric cancer

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Subject headings matrix metalloproteinase-2 mRNA; stomach neoplasms; polymerase chain reaction

INTRODUCTION

During tumor invasion and metastasis, malignant cells in primary site acquire the ability to degrade extracellular matrix (ECM) and penetrate tissue barriers. Among the proteolytic enzymes which degrade ECM, matrix metalloprotenase (MMP) is one of the important ones. MMP_2 (72kDa type IV collagenase) is a member of the MMPs gene family which degrades the macromolecules of connective tissue and ECM, such as collagen, proteoglycans, laminin and fibronectin. Thus MMP_2 is believed to play an important role in tumor invasion and metastasis. Several imm unohistochemical studies have shown that MMP_2 mRNA is overexpressed in gastric cancer and related to the clinical stage of cancer[1,2]. However, samples were not enough and lack completeness in these studies. Reverse trans criptase-polymerase chain reaction (PT-PCR) was used in our study to examine the expression of MMP_2 mRNA in tumor and normal tissues adjacent to human gastric cancer.

MATERIALS AND METHODS

Samples

The samples, including tumour tissue and tumour-adjacent normal tissue (2 cm and 5 cm or over 5 cm distance from the tumour) came from twenty gastric cancer patients at the surgery department of our hospital and People's Hospital of Yun-He County, Zhejiang. Gastric tissues from five benign ulcer patients after partial gastrectomy were used as controls. The histological diagnosis was made by the pathologists of these two hospitals. Patients' clinical features are shown in Table 1. Among these cancer patients, five and fifteen were cases of early and advanced cancer respectively. All samples were quenched in liquid nitrogen immediately after operation and were then stored at -70℃ until used for the study.

Reagents and primer synthesis

The dNTP, RNAsin and MMLV reverse transcriptase and Taq DNA were provided by Str atagene, La Jolla, CA, U.S.A. MMP_2 primer pair was synthesized by Shanghai Cell Research Institute, Chinese Academy of Sciences[2] and its sense and anti-sense were 5'-ACAAAGAGTGGCAGTGCAA-3' and 5'-CACGAGCAAAGGCATCATCC-3' respectively. The expected size of MMP-2 product was 302bp.

RT-PCR analysis

Total RNA was extracted from frozen tissues by cesium chloride purifing method. A total amount of 20 μL reaction solution contained 5 μg RNA sample tissue, 1 mmol/L dNTP, 10 U RNAasin, 100 mmol/L Tris-HCl pH 8.4, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 100 mg/mL BSA, 100 pmol-random six-polyoligo-nucleotide and 100 U MMLV reverse transcriptase. The reverse transcriptional condition was 37 ℃ for 1 h, and 95 ℃ for 5 min. Twenty μL cDNA reverse transcriptase product was put in PCR reaction solution containing 100 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 100 mg/mL BSA, 30 pmol sense and anti-sense primers, and then 2 U Taq DNA polymerse was added in the solution. The PCR amplification condition was: denatured at 95 ℃ for 1 min, annealed at 65 ℃ for 1 min and extended at 72 ℃ for 1min. The number of cycles was 35.10 μL DNA, amplificating product was subjected to electrophresis in 4% agarose gel, stained with ethidium bromide and observed under ultroviolet light. The photographs of PCR results were used to measure the level of optical density (OD) of MMP-2 cDNA bands with densitometry (Backman CD 2000).

Statiscal analysis

The significance of differences in expression rates and OD levels among groups was determined by χ^2 test and Student's *t* test respectively.

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RESULTS

Expression of MMP-2 mRNA in tumor and tumor-adjacent tissues (Figure 1)

In 20 cases of gastric cancer, MMP_2 mRNA was expressed in 13 tumor tissues, 11 in 2 cm and 6 in ⩾ 5cm adjacent tissues respectively (Table 1). The positive rate of MMP^2 mRNA expression in tumor tissues was significantly higher than that in ⩾5cm adjacent tissues (P<0.05). There was no positive expression of MMP^2 mRNA in the 5 samples of the control group.

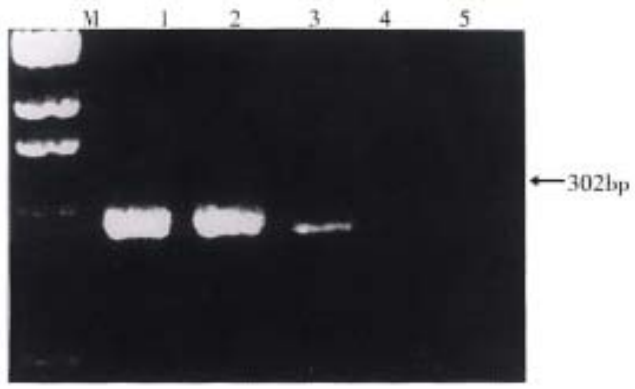

Figure 1 Expression of MMP-2 mRNA in case No.3
Lane M: Marker; Lane 1, 2: tumor tissues; Lane 3-5: 2cm, ⩾5cm adja cent tissues and normal gastric tissue of one control.

Table 1 Clinical features of cases and expression of MMP-2 mRNA

No.	Age/sex	Location	Histology	Clinical stage[b]	MMP2 mRNA expression		
					T	2cm	⩾5cm
1[a]	64/M	Pylorus	Poor	T1N0M0(I)	-	-	-
2	48/M	Body	Poor	T4N0M0(IV)	+	+	-
3	72/F	Lesser curve	Poor	T4N2M1(IV)	+	+	-
4	75/F	Lesser curve	Poor	T3N0M0(II)	+	+	-
5	71/M	Body	Poor	T2N1M0(IVA)	+	+	-
6	63/M	Lesser curve	Well	T4N2M0(IV)	-	-	+
7[a]	73/F	Lesser curve	Poor	T1N1M0(IIIA)	+	-	-
8[a]	46/M	Lesser curve	Poor	T1N0M0(I)	-	-	-
9	60/F	Body	Poor	T3N0M0(II)	+	-	-
10	57/M	Pylorus	Poor	T3N1M1(IV)	+	+	+
11	53/M	Body	Poor	T4N1M0(IV)	+	+	-
12[a]	72/F	Antrum	Poor	T1N0M0(I)	-	-	-
13	59/M	Body	Poor	T3N2M0(IIIB)	+	-	-
14[a]	38/M	Antrum	Well	T1N0M0(I)	-	-	-
15	66/F	Cardia	Poor	T3N1M0(IIIA)	+	+	+
16	55/M	Antrun	Well	T2N0M0(IB)	-	-	-
17	74/M	Body	Poor	T4N2M1(IV)	+	+	+
18	67/M	Cardia	Well	T3N2M0(IVB)	-	-	-
19	67/F	Body	Poor	T4N2M1(IV)	+	+	+
20	56/M	Body	Poor	T4N2M1(IV)	+	+	+

T: Tumor tissues; 2cm: 2cm adjacent tissue from tumor; ⩾5cm: 5cm or over 5cm adjacent tissue from tumor. [a]: early gastric cancer. Poor: poorly differentiated adenocarcinoma; Well: well differentiated a denocarcinoma. [b]: According to the American Joint Commission Staging of Gastric Cancer.

The OD levels of MMP-2 mRNA in tumor and tumor-adjacent tissues (Figure 2)

The OD of MMP-2 detected cDNA signals ranged from 1.10 to 19.23 (mean 5.38± 0.98) in tumor tissues, 0.86 to 4.17 (mean 2.41±0.30) in 2 cm and 0.78 to 3.80 (mean 1.88±0.22) in ⩾5 cm adjacent tissues respectively. There was significant difference in OD levels between tumor tissues group and 2 cm or ⩾5cm tumoradjacent tissues one (P < 0.01), and no si gnificant difference in OD levels between the two adjacent tissues groups.

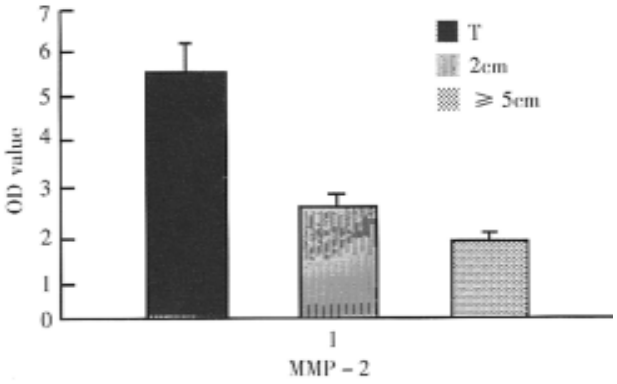

Figure 2 OD of MMP-2 cDNA signals in tumor tissues and tumor-adjacent tissues (2cm and ⩾5cm). As compared with tumor tissues: 2cm: P<0.01; ⩾5cm: P<0.01.

The OD levels of MMP-2 mRNA in early and advanced cancer (Table 2)

The MMP-2 cDNA signals in tumour and 2cm adjacent tissues of 15 advanced cancer were significantly higher than those in the two corresponding tissues of 5 early cancer respectively (P<0.05). In the ⩾5 cm adjacent tissues, there was no-significant difference in the signals between the advanced and early cancer.

Table 2 OD of MMP-2 cDNA signals in early and advanced cancer ($\bar{x}\pm s_{\bar{x}}$)

	T	2cm	⩾5cm
Early cancer (n=5)	1.63±0.42	1.07±0.29	1.50±0.22
Advanced cancer (n=15)	6.63±1.12[a]	2.80±0.34[a]	1.94±0.29

[a]P<0.05, *vs* early cancer.

DISCUSSION

In 20 gastric cancer cases, 13, 11 and 6 cases positively expressed MMP-2mRNA in tumor, 2 cm adjacent and ⩾5 cm adjacent tissues respectively. The cases with MMP-2 mRNA expression in tumor tissues and their corresponding tumor adjacent normal tissues (2 cm and/or ⩾5 cm) were almost poorly differentiated adenocarcinoma and in higher clinical stage. MMP-2 mRNA was seldom expressed in tumor and tumor adjacent tissues of well differentiated or early carcinoma. These results

showed that proliferative and invasive gastric cancer cells had higher MMP-2 secretion. In ultrastructural study, MMP-2 mRNA was expressed markedly in cancer cells with rich false feet and rapid movement in culture, but insignificantly or with few false feet in cancer cells from unmetastatic and uninvasive gastric cancerous tissues, indicating that MMP-2 secretion was correlated with the invasion and metastasis of gastric cancer[3]. In addition, some immunohistochemistry studies have shown that the positive rate of staining cells for MMP-2 protein was consistently higher in poorly differentiated and diffuse gastric carcinoma than that in well differentiated and early gastric carcinoma[1,4]. The results of these studies were very similar to those of MMP-2 mRNA expression in our study, indicating that the increased MMP-2 positive staining was related to the overexpression of MMP-2 mRNA. This is probably due to increase in MMP-2 transcriptional activity and MMP-2 protein products in the cell proliferative process of gastric cancer which is often accompanied with the acceleration of cancer invasion and metastasis.

In the current study, although the levels of MMP-2 cDNA signals in tumor-adjacent tissues (2 cm and/or $\geqslant$5 cm) were lower than those in tumor tissues, MMP-2 mRNA was overexpressed in the tumor adjacent tissues in certain extent, suggesting that both gastric cancer cells and adjacent mesenchymal cells, including fibrocyte, endothelium cell, macrophage and lymphocyte have the ability to secrete MMP-2. There may be information exchange between the cancer cells and these mesenchymal cells through the dissolvable intercellular substance and membrane cement factor, and such information exchange may regulate the productio n of MMP-2. This may be very important in elucidating the mechanism of invasion and metastasis of cancer cells[5,6]. In our case No. 6, MMP-2mRNA was detected only in tumor-adjacent tissues, but not in tumor tissue. The reason for this is unclear. The discrepancy may be due to the necrotic tumor tissue. Overexpression of MMP-2 mRNA only in tumour adjacent tissues also indicates the malignant degree of cancer is rather high.

The prognosis of early gastric cancer is better than that of advanced cancer. Our study showed that the levels of MMP-2 cDNA signal in advanced cancer tissues (tumor and 2 cm tumor-adjacent tissues) were significantly higher. This suggests MMP-2 may play a role in gastric cancer invasion and metastatic progression, and the overexpression may be associated with poor prognosis. Further study on relationship between the expression of MMP-2 mRNA and the survival rate of gastric cancer is being carried out.

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Islet separation and islet cell culture *in vitro* from human embryo pancreas

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Subject heading cell culture; islet cell; islets of langerha ns; transplantation; pancreas/embryology

INTRODUCTION

Diabetes mellitus is the most common disease and its death rate ranks the 8th in the world. Up to now, its incidence has a tendency to increase[1]. Since disorder of sugar metabolism might result in microvascular degeneration and injury of important human organs, it will endanger human health seriously. In 1969, Younszai first reported the method that could decrease diabetes symptoms by islet tissue transplantation. In 1981, our country began to treat diabetes type I by transplanting cultured islet tissues. In our study, we digested the pieces of pancreas with collagenase and made morphological observation on islet cells cultured for 3 d, 5 d, 7 d, 9 d and 13 d respectively, and the contents of insulin and C-peptide in the supernatant were detected by radioimmunoassay. The experimental model of rabbit diabetes was established, and certain curative effect was achieved in the treatment of experimental diabetes in rats.

METERIALS AND METHODS

Islet isolation

Four cases of 18wk-28wk human embryo induced by hydrostatic bag were sterilized with 75% alcohol, and their pancreas were removed with the surrounding connective tissues eliminated. After the pancreas were washed with cold Hank's balanced salt solution, they were cut into 1 mm fragments digested three times with 0.5 g/L collagenase (Sigma Type V 663 U/mg) and shaken thoroughly. Then the islets were carefully isolated under stereoscope (islets are white with different dimensions), and washed two times with Hank's balanced salt solution and put into glass bottles to be cultured.

Islet cells culture

Islet cells were cultured with RPMI-1640 medium containg 20% bovine serum, 10 mmol/L glutamine, 80 U penicillin and 0.5 g streptomycin. Appro ximately 30 islets were inoculated in the 5mL bottles, and were cultured in CO_2 incubator (95% atmosphere, 5% CO_2, 37 ℃). The cells were digested by 0.25% sodium citrate and their fibroblast was cleaned[2]. The medium solution was replaced every 2 d after the 3 days. One mL-2 mL culture solution was put into clean bottles which were placed into refrigerater (4 ℃) to detect the contents of insulin and C-peptide.

Observation of islet cell morphology

In the process of culture, growth of islet cells was observed under invert microscope, and the islet cells cultured for 3 d, 5 d, 7 d, 9 d and 13 d respectively were observed under transmission electron microscope. The samples were fixed with 1.25% glutaraldehyde, and embedded with epoxy 618, sectioned with LKB-V ultramicrotome, and observed under JEM-100CX electron microscope.

Measurement of content insulin and C-peptide by radioimmunoassay

FT-630G computer with multiprobe γ counter and kit of De Pu Company were used to collect the supernatant fluid of islet cells cultured for 3 d, 5 d, 7 d, 9 d and 13 d respectively by strict standard operation, and contents of insulin and C-peptide in the culture suspension were measured by radioimmunoassay (RIA) with antigen labeled by radionuclide ^{125}I.

Establishment of experimental model of rabbit diabetes and evaluation of experimental cure

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effect

Fasting blood sugar, insulin and C peptide were detected in 18 adult rabbits (9 male and 9 female weighing 1.5 kg - 2.0 kg). All rabbits were given injection of alloxan of 150 mg/kg into the posterior auricular vein to establish the model of rabbit experimental diabetes. Their biological behavior was observed after 48 h, and the contents of blood sugar, insulin and C- peptide were measured[3]. The cultured rabbit embryo islet cells were injected (1.1×10^7 cells/each) into their pancreatic artery after 72 h and the experimental treatment was evaluated.

RESULTS

Morphology of the cultured islet cells

After the islets were cultured for 24 h, fibroblast growth was found on the wall of glass bottle, but no islet cell mass was found. After the fibroblasts were eliminated with citrate sodium, islet cells began to grow and form a single layer of cells with typical morphology. Under invert microscope, the cells were found growing quite well, most of them were of epithelioid type with plenty of cytoplasm. The cell number was counted directly after stained with trypan blue and their survival rate reached up to 90%. Many cells were observed under transmission electron microscope and most of them were found to be beta cells with m any β-granules, alpha cells, and a few extracrinous cells and macroph0ages. These beta cells after cultured for 5 d-9 d with a high density cytoplasm and cytoplasmic β-granule developed well. But eleven days later, the number of cytoplasmic granules decreased with karyopyknosis and degeneration in them.

Contents of insulin and C-peptide in human embryo islet cell culture solution (Table 1)

Table 1 Contents of insulin and C-peptide in human embryo islet cel l culture solution

Islet cells (culture days)	Contents of insulin (IU/L)	Contents of C-peptide (mg/L)
3	47.30	3.05
5	64.75	6.05
7	72.30	6.20
9	72.70	>6.00
11	68.75	5.15
13	72.20	>6.00

*Insulin antibody is negative in all tubes.

Contents of blood sugar, insulin and C-peptide in normal rabbits and diabetes rabbits after experimental treatment (Table 2)

Table 2 Changes of blood sugar, insulin, C-peptide after experiment al treatment in normal and diabetes rabbits ($\bar{x}\pm s$)

Experimental rabbits	Blood sugar (g/L)	Contents of insulin(IU/L)	Contents of C-peptide (mg/L)
Normal controls	1.0524±0.0510	6.27±4.6	13.23±4.77
Diabetes rabbits	4.7979±0.9233	2.65±1.4	Not detected
Experimentally treated rabbits	3.3193±0.4110[a]	13.88±1.5[a]	Not detected
P	<0.01	<0.05	

[a]After transplanted with rabbit islet cells, diabetes rabbits showed good spirit, less drinking and urine excretion.

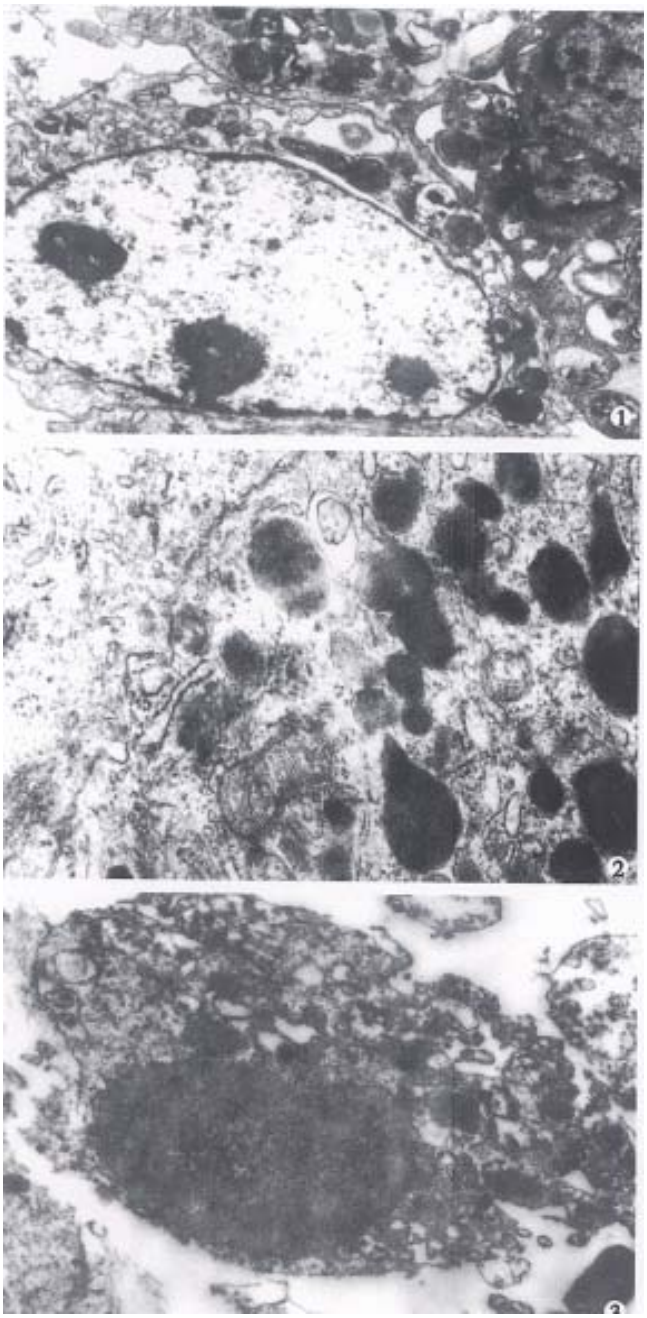

Figure 1 Islet cells developed well after cultured for 5d. TEM ×10 000

Figure 2 Cytoplasmical APUD granules were observed after cultured for 9d. TEM×20000

Figure 3 Islet cells were aging after cultured for 13d with karyopyknosis and cytoplasmic lysis. TEM×30 00

DISCUSSION

The quality and quantity of islet cells play a crucial role in the effectiveness of transplantation. An ideal preparative method can provide sufficient pure, viable and functional islet cells. We have achieved good digestive effect by digesting pancreas with collagenase at optimum pH and temperature[4] . In order to obtain good islets, it is necessary to control digestive time and mix the pieces of pancreas with collagenase thoroughly. The islet cells obtained were growing and their structure was not mature before they were cultured for 5 d. But after 5 d-9 d, they grew well with their structure fully matured. The survival rate was 90% according to the trypan blue stainning. However, eleven days later, most cells became ageing with karyopyknosis and cytoplasmiclysis.

Activity and function are the important indexes in assessing the effect of islet cells. The contents of insulin and C-peptide in the culture suspension measured by radioimmunoassay were two times higher than those in normal control serum, indicating that our method is rather good. The highest amount of contents of insulin and C-peptide was found after they were cultured for 13 d, but was not in accordance with the morphological data. The reason why their contents inc reased in insulin culture solution was aging and released the remaining insulin that made insulin contents of the degeneration of most cells and release of the remaining insulin from cells from the 11th day.

We have used the same method to culture rabbit islet cells and made experimental treatment of rabbit diabetes model after rabbit islet cells were cultured for 7 d. Blood sugar was 1.052 g/L ± 0.5012 g/L and 4.7979 g/L ± 0.9233 g/L in normal and experimental rabbits. Three days after the rabbits were given an injection via pancreatic artery at a density of 1. 1×10^7 cells/each islet cell, their blood sugar decreased to 3.3193 g/L ± 0.4110 g/L. Compared with diabetes rabbit ($P <$ 0.01), the difference was significant. Serum insulin contents increased from 2.65 IU/L ± 1.4 IU/L to 13.88IU/L±1.5IU/L (P<0.05), the difference was also significant. All these indicated that experimental treatment was effective.

In the past ten years, most tissue transplantation focused on subcutaneous and intramuscular transplantation of cultured islets. Present method is to treat diabetes type I with cultured islet cells which were planted by portal vein[5].

Therefore, we believe that the culture of the islet cells by this method can be used to treat diabetes type I, and the optimum time of transplantation is 5-9 days after the islet cells were cultured.

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