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• REVIEW •

Key role of mast cells and their major secretory products in inflammatory bowel disease

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Abstract

Historically, mast cells were known as a key cell type involved in type I hypersensitivity. Until last two decades, this cell type was recognized to be widely involved in a number of non-allergic diseases including inflammatory bowel disease (IBD). Markedly increased numbers of mast cells were observed in the mucosa of the ileum and colon of patients with IBD, which was accompanied by great changes of the content in mast cells such as dramatically increased expression of TNFα, IL-16 and substance P. The evidence of mast cell degranulation was found in the wall of intestine from patients with IBD with immunohistochemistry technique. The highly elevated histamine and tryptase levels were detected in mucosa of patients with IBD, strongly suggesting that mast cell degranulation is involved in the pathogenesis of IBD. However, little is known of the actions of histamine, tryptase, chymase and carboxypeptidase in IBD. Over the last decade, heparin has been used to treat IBD in clinical practice. The low molecular weight heparin (LMWH) was effective as adjuvant therapy, and the patients showed good clinical and laboratory response with no serious adverse effects. The roles of PGD2, LTC4, PAF and mast cell cytokines in IBD were also discussed. Recently, a series of experiments with dispersed colon mast cells suggested there should be at least two pathways in man for mast cells to amplify their own activation-degranulation signals in an autocrine or paracrine manner. The hypothesis is that mast cell secretogogues induce mast cell degranulation, release histamine, then stimulate the adjacent mast cells or positively feedback to further stimulate its host mast cells through H_1 receptor. Whereas released tryptase acts similarly to histamine, but activates mast cells through its receptor PAR-2. The connections between current anti-IBD therapies or potential therapies for IBD with mast cells were discussed, implicating further that mast cell is a key cell type that is involved in the pathogenesis of IBD. In conclusion, while pathogenesis of IBD remains unclear, the key role of mast cells in this group of diseases demonstrated in the current review implicates strongly that IBD is a mast cell associated disease. Therefore, close attentions should be paid to the role of mast cells in IBD.

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INTRODUCTION

Historically, mast cells were known as a key cell type involved in type I hypersensitivity[1]. Until last two decades, this cell type was recognized to be widely involved in a number of non-allergic diseases in internal medicine including chronic obstructive pulmonary disease (COPD), Crohn's disease, ulcerative colitis, liver cirrhosis, cardiomyopathy, multiple sclerosis and rheumatoid arthritis, *etc*. (Table 1). This article will focus solely on the relationships between mast cells and inflammatory bowel disease, give evidence for a hypothesis of self-amplification mechanism of mast cell degranulation in gut and discuss the potential therapies for the treatment of inflammatory bowel disease (IBD).

Table 1 Mast cells involved in non-allergic diseases in internal medicine

Disease	Evidence
Chronic obstructive pulmonary disease (COPD)	Mast cell hyperplasia in epithelia and bronchial glands[2,3], tryptase and histamine release in BALF[4]
Cor pulmonale	Mast cell hyperplasia in bronchial and vascular tissues[5]
Bronchiectasis	Increased numbers of degranulated mast cells in lung tissue, and higher tryptase concentrations in BALF[6]
Acute respiratory distress syndrome (ARDS)	Mast cell hyperplasia[7] and degranulation[8]
Bronchiolitis obliterans-organizing pneumonia	Mast cell hyperplasia[9] and degranulation[10]
Cystic fibrosis	Mast cell hyperplasia[11] and degranulation[12] in lung
Interstitial lung diseases	Mast cell hyperplasia[13] and degranulation[14]
Silicosis	Mast cell hyperplasia[15]
Sarcoidosis	Mast cell hyperplasia[16], and degranulation[17] in lung
Lung cancer	Mast cell hyperplasia[18]
Tuberculosis	Mast cell degranulation[19]
Gastritis	Mast cell hyperplasia[20] and degranulation[21]
Peptic ulcer	Mast cell hyperplasia[22] and degranulation[23]
Hepatocellular carcinoma	Mast cell hyperplasia[24]
Ulcerative colitis	Mast cell hyperplasia[25,26] and degranulation[27]
Crohn's disease	Mast cell hyperplasia[28] and degranulation[29]
Liver cirrhosis	Mast cell hyperplasia[30]
Hepatitis	Mast cell hyperplasia[31,32]
Pancreatitis	Mast cell hyperplasia and degranulation[33]
Atherosclerosis	Mast cell hyperplasia[34]
Myocardial infarction	Mast cell hyperplasia and degranulation[35,36]
Congenital heart disease	Mast cell hyperplasia and subtype change[37]
Myocarditis	Mast cell hyperplasia[38]
Cardiomyopathy	Mast cell hyperplasia[39] and degranulation[40]
Diabetes	Mast cell hyperplasia[41]
Thyroiditis	Mast cell hyperplasia[42]
Osteoporosis	Mast cell hyperplasia[43]
Glomerulonephritis	Mast cell hyperplasia[44]
Nephropathy	Mast cell hyperplasia[45]
Multiple sclerosis	Mast cell hyperplasia[46]
Rheumatoid arthritis	Mast cell degranulation[47]
Osteoarthritis	Mast cell hyperplasia[48]
Rheumatic arthritis	Mast cell hyperplasia[49]

MAST CELLS

Morphology

Mast cell is the cell that contains numerous metachromatically stained basophilic granules in its cytoplasm. It has various sizes between species with diameters of up to 30 μm reported in humans, and from 3.5 μm to 22 μm in rodents[28,50]. In human lung, for example, the range of mast cell diameters has been reported to be between 9.9 μm and 18.4 μm[51] and in human skin between 4 μm and 18 μm[52]. The shape of mast cell varies as well, it has been described as polyhedral, fusiform, ovoid, and rectangular, and appears dependent on tissue locations. Mast cell nuclei are usually round or oval and have peripherally dispersed heterochromatin[53].

Up to 40% of the volume of mast cell is occupied by membrane-enclosed secretory granules[54]. There are 50 to 500 secretory granules in one mature human mast cell, each with a diameter ranging from 0.2 to 0.5 μm. Within a given mast cell, these granules are usually of a uniform size, but there is variability from cell to cell[55]. Mast cell granules originate from the Golgi apparatus, which is responsible for the synthesis and organization of the preformed mediators contained therein[56].

Mediators

Upon activation mast cell can release its mediators to fulfill its biological functions. Among preformed mediators, histamine is a primary amine synthesized from histidine in the Golgi apparatus, from where it is transported to the granule for storage in ionic association with the acidic residues of glycosaminoglycans side chains of heparin and proteinases[57,58]. The histamine content of mast cells dispersed from human lung and skin is similar at 2 to 5 pg/cell, and the stored histamine ranges from 10 to 12 μg/g in both tissues[53]. As only mast cell and basophil contain histamine in man, and few basophils in human tissue histamine can be used as a marker of mast cell degranulation.

Proteoglycans in human mast cells include heparin and chondroitin sulphate, which contains several highly sulphated glycosaminoglycan side chains attached to a single chain protein core. They comprise the major supporting matrix of the mast cell granule with the sulphate groups binding to histamine, proteinases and acid hydrolases.

Neutral proteases of mast cells are also preformed mediators. Three mast cell unique neutral proteinases (tryptase, chymase and carboxypeptidase) have been isolated in man and there is evidence also for a proteinase with antigenic and enzymatic properties similar to those of neutrophil cathepsin G in mast cells[59,60]. Mast cell tryptase, chymase and carboxypeptidase are reliable markers of mast cell degranulation. Based on their content of proteinases, mast cells can be classified into two types in man, with MC_T cells defined as those containing tryptase but not chymase, and MC_{TC} cells as those containing both tryptase and chymase[61]. Subsequently both carboxypeptidase[62] and cathepsin G like proteases[63,64] have been found to be localised exclusively in the MC_{TC} population.

Newly generated mediators include eicosanoids and platelet activating factor (PAF). Eicosanoids are a group of newly generated mediators of mast cells. Immunological activation of mast cells results in the liberation of arachidonic acid from phospholipids in the cell membrane. This 20-carbon fatty acid is then rapidly oxidized along either of two independent pathways, namely the cyclooxygenase pathway to form PGD_2 and the lipoxygenase pathway to form LTC_4. These are the only two eicosanoids produced by human mast cells[65]. PAF is also a product of phospholipid metabolism in mast cells.

Mast cell cytokines may constitute a third category in that they may be both preformed and newly synthesized. For instance, it has been reported that approximately 75%, 10%, 35%, and 35% of mast cells contain IL-4, IL-5, IL-6 and TNFα, respectively in the nasal mucosa and bronchus[66]. Mast cell contains also IL-1β, IL-3, IL-8, IL-9, IL-10, IL-13, IL-16, IL-18, IL-25, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor macrophage chemotactic peptide (MCP)-1, 3, 4, regulated on activation of normal T cell-expressed and secreted protein (RANTES) and eotaxin[67].

Mast cell activation

Mast cell activation is a crucial step in mast cell involved events because it seems that only activated mast cells are able to cause pathophysiological changes. There are a number of compounds that can activate mast cells. They are antigens, anti-IgE and ionophores. Skin mast cells but not those of lung, tonsil or gut can be activated by other diverse compounds including substance P, VIP, C5a and C3a, somatostatin, compound 48/80, morphine, pepstatin, MBP, PAF, platelet factor 4 and very-low-density lipoproteins[68,53]. Stem cell factor[69], eosinophil cationic protein[70] and tryptase[71] have also been found to be able to activate human mast cells.

The mechanisms of mast cell activation differ with different classes of triggers. Human skin mast cells are able to respond to non-crosslinking stimuli, such as neuropeptides, morphine, and complement fragments[68]. IgE-dependent mast cell activation is a complicated process. It involves a specific IgE bound to its high affinity receptor (FcεR1) on the surface of mast cells, a multivalent antigen (Ag) crosslinking specific IgEs bound to FceR1 and a signal transduction and translation process in mast cells.

Models for mast cell degranulation

While normal human mast cell line is not available, dispersed and purified mast cells are essential for investigating mast cell functions. Human mast cells have been dispersed from skin, lung, tonsil, synovium heart and intestine tissues by incubation with collagenase and hyaluronidase. These dispersed cells have appreciable morphological and functional properties of mast cells. Since dispersed cells can be evenly distributed in experiment, it is the most popular method at present. However, the purity of mast cells with this system is only 0.5-10% depending on tissues.

Chopped tissue fragments were also used for mast cell degranulation study. We found that it was difficult to evenly distribute cells in tissue fragments, therefore causing large experimental errors.

Laboratory animal tissues or mast cells are widely used in mast cell degranulation study. Thus, rat and mouse peritoneum, guinea pig lung and cultured mouse bone marrow derived mast cells are the most popular models. However, it is always adequate to use human mast cells to investigate the pathophysiological process of human disease.

INCREASED NUMBERS OF MAST CELLS IN IBD

As early as in 1980, Dvorak and colleagues[28] reported that the number of mast cells was markedly increased in the involved area of the ileum of patients with Crohn' s disease. In 1990, Nolte *et al*.[72] found that the mast cell count in patients with ulcerative colitis was increased compared with that in control subjects and patients with Crohn' s disease. In an individual patient, the mast cell count obtained from inflamed tissue was greater than that of normal tissue. This finding was taken further by King *et al*.[26] in 1992 that the number of mast cells in active ulcerative colitis was 6.3 in active inflammation, 19.5 at the line of demarcation and 15.8 in normal mucosa. The accumulation of mast cells at the visible line of demarcation between normal and abnormal mucosa suggested that mast cells played a crucial role in the pathogenesis of the disease, either causing further damage or limiting the expansion of damage. Recently, a report[73] provided us an even more convincing evidence and clear picture on the elevated number

of mast cells in inflammatory bowel disease. Nishida and colleagues found that there were greater numbers of mast cells than macrophages in the lamina propria of patients with inflammatory bowel disease though this was not found in patients with collagenous colitis. Interestingly, increased numbers of mast cells were observed throughout the lamina propria, particularly in the upper part of lamina propria, whereas increased numbers of macrophages were only seen in the lower part of lamina propria in patients with inflammatory bowel disease. This could result from that accumulated mast cells released their proinflammatory mediators, and these mediators, at least tryptase[74] and chymase[75], induced macrophage accumulation in the lower part of lamina propria. Dramatically increased numbers of mast cells were also observed in the hypertrophied and fibrotic muscularis propria of strictures in Crohn' s disease compared with normal bowel ($81.3/mm^2$ *vs* $1.5/mm^2$)[76].

Not only the number of mast cells was elevated[77], but also the contents of mast cells were greatly changed in inflammatory bowel disease in comparison with normal subjects. Laminin, a multi-functional non-collagenous glycoprotein, which is normally found in extracellular matrix was detected in mast cells in muscularis propria (but not those in submucosa), indicating that mast cells may be actively involved in the tissue remodeling in Crohn' s disease[76]. Similarly, the number of TNF-α positive mast cells was greater in the muscularis propria of patients with Crohn' s disease than that in normal controls[78]. In the submucosa of involved ileal wall of Crohn' s disease, more TNF-α positive mast cells were found in inflamed area than uninflamed area. Since those TNF-α positive mast cells were mail cell type that expressed TNF-α in ileal wall, the successful treatment of Crohn' s disease with anti-TNF-α antibody could well be the consequence that the antibody neutralized the excessively secreted TNF-α from mast cells. This indirectly proved the important contribution of mast cells to the development of Crohn' s disease. Increased number of IL-16 positive mast cells, which was correlated well with increased number of $CD4^+$ lymphocytes, was also observed in active Crohn' s disease[79], indicating that this chemokine may selectively attract $CD4^+$ lymphocytes to the involved inflammatory area[80,81]. In chronic ulcerative colitis, increased number of substance P positive mast cells was observed in gut wall, particularly in mucosa[82], indicating the possibility of neuronal elements being involved in the pathogenesis of the disease.

Increased number of mast cells was also seen in a number of diseases closely related to inflammatory bowel disease. Primary sclerosing cholangitis and chronic sclerosing sialadenitis showed similar marked mast cell infiltration pattern with inflammatory bowel disease[83]. Focal active gastritis is a typical pathological change in Crohn' s disease[84], in which large number of mast cells accumulate at the border of the lesions[20]. In the animal models, increased number of mast cells in gastrointestinal tract was observed in dogs with inflammatory bowel disease in comparison with healthy dogs[85]. When given 3% dextran sulphate sodium for 10 days[86] or water avoidance stress for 5 days[87], pathological changes such as mucosal damage and edema were developed in rats, and these were accompanied by mast cell hyperplasia and activation. However, the same treatment had little effect on mast cell deficient Ws/Ws rats, implying the importance of mast cells in the development of inflammatory bowel disease.

EVIDENCE OF MAST CELL DEGRANULATION IN IBD

As early as in 1975, Lloyd and colleagues observed that there were marked degranulation of mast cells and IgE-containing cells in the bowel wall of patients with Crohn' s disease[29], and this observation later became an important investigation area for understanding the pathogenesis of Crohn' s disease. In 1980, Dvorak *et al*. described in more detail the degranulation of mast cells in the ileum of patients with Crohn' s disease[28] with transmission electron microscopy technique. Similarly, with electron microscopy technique, degranulation of mast cells was seen in the intestinal biopsies of patients with ulcerative colitis[88]. Using immunohistochemistry technique with antibodies specific to human tryptase or chymase, both of which are exclusive antigens of human mast cells, mast cell degranulation was found in the mucosa of bowel walls of patients with Crohn' s disease, ulcerative colitis[89] and chronic inflammatory duodenal bowel disorders[90].

INVOLVEMENT OF HISTAMINE IN PATHOGENESIS OF IBD

Using segmental jejunal perfusion system with a two-balloon, six channel small tube, Knutson and colleagues found that the histamine secretion rate was increased in patients with Crohn' s disease compared with normal controls, and the secretion of histamine was related to the disease activity, indicating strongly that degranulation of mast cells was involved in active Crohn' s disease[91]. The highly elevated mucosal histamine levels were also observed in patients with allergic enteropathy and ulcerative colitis[27]. Moreover, enhanced histamine metabolism was found in patients with collagenous colitis and food allergy[92], and increased level of N-methylhistamine, a stable metabolite of the mast cell mediator histamine, was detected in the urine of patients with active Crohn' s disease or ulcerative colitis[93,94]. Since increased level of N-methylhistamine was significantly correlated to clinical disease activity, the above finding further strongly suggested the active involvement of histamine in the pathogenesis of these diseases.

Interestingly, mast cells originated from the resected colon of patients with active Crohn' s disease or ulcerative colitis were able to release more histamine than those from normal colon when being stimulated with an antigen, colon derived murine epithelial cell associated compounds[95]. Similarly, cultured colonrectal endoscopic samples from patients with IBD secreted more histamine towards substance P alone or substance P with anti-IgE than the samples from normal control subjects under the same stimulation[96]. In a guinea pig model of intestinal inflammation induced by cow' s milk proteins and trinitrobenzenesulfonic acid, both IgE titers and histamine levels were higher than normal control animals[97].

As a proinflammatory mediator, histamine is selectively located in the granules of human mast cells and basophils and released from these cells upon degranulation. To date, a total of four histamine receptors H_1, H_2, H_3 and H_4 have been discovered[98] and the first three of them have been located in human gut[99,100], proving that there are some specific targets on which histamine can work in intestinal tract. Histamine was found to cause a transient concentration-dependent increase in short-circuit current, a measure of total ion transport across the epithelial tissue in gut[101]. This could be due to the interaction of histamine with H_1-receptors that increased Na and Cl ions secretion from epithelium[102]. The finding that H_1-receptor antagonist pyrilamine was able to inhibit anti-IgE induced histamine release and ion transport[103] suggests further that histamine is a crucial mediator responsible for diarrhea in IBD and food allergy. The ability of SR140333, a potent NK1 antagonist in reducing mucosal ion transport was most likely due to its inhibitory actions on histamine release from colon mast cells[104].

INVOLVEMENT OF MAST CELL PROTEASES IN PATHOGENESIS OF IBD

Tryptase is a tetrameric serine proteinase that constitutes some

20% of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells[105] in a catalytically active form[106]. The ability of tryptase to induce microvascular leakage in the skin of guinea pig[107], to accumulate inflammatory cells in the peritoneum of mouse[74] and to stimulate release of IL-8 from epithelial cells[108], and the evidence that relatively higher secretion of tryptase has been detected in ulcerative colitis[109] implicated that this mediator is involved in the pathogenesis of intestinal diseases. However, little is known about its actions in IBD. Recently, proteinase activated receptor (PAR)-2, a highly expressed receptor in human intestine[110] was recognized as a receptor of human mast cell tryptase[111]. Colonic administration of PAR-2 agonists up-regulated PAR-2 expression, induced granulocyte infiltration, colon wall edema and damage and stimulated an increased paracellular permeability of colon mucosa[112]. PAR-2 agonists were also able to stimulate intestinal electrolyte secretion[113]. Interestingly, some 60% and 46% of mast cells in ulcerative colitis tissues expressed PAR-2 or TNF-α, respectively. PAR-2 agonists were able to stimulate TNF-α secretion from mast cells[114] and secreted TNF-α could then enhance PAR-2 expression in a positive feedback manner[113]. These findings indicated further the importance of TNF-α and mast cells in the pathogenesis of IBD.

Chymase is a serine proteinase exclusively located in the same granules as tryptase and could be released from granules together with other preformed mediators. Large quantity of active form chymase (10 pg per mast cell) in mast cells[115] implicates that this mast cell unique mediator may play a role in mast cell related diseases. Indeed, chymase has been found to be able to induce microvascular leakage in the skin of guinea pig[116], stimulate inflammatory cell accumulation in peritoneum of mouse[75], and alter epithelial cell monolayer permeability *in vitro*[117]. However, little is known about its actions in IBD. Mast cell carboxypeptidase is a unique product of MC_{TC} subtype mast cells. There is some 10 pg in each mast cells. No information about the relationship between mast cell carboxypeptidase and IBD is available, but the successful coloning of mast cell carboxypeptidase from human gut tissue and obtaining of recombinant human mast cell carboxypeptidase [chen 2004] will certainly help to initiate the investigation of the role of mast cell carboxypeptidase in IBD. It is astonished to learn the fact that the potential roles of mast cell neutral proteinases in IBD have been almost completely ignored till today. Since they are the most abundant granule products of mast cells and have been demonstrated to possess important actions in inflammation, they should certainly contribute to the occurrence and development of IBD.

INVOLVEMENT OF HEPARIN IN PATHOGENESIS OF IBD

Over the last decade, heparin, a unique product of human mast cells and basophils, has been used to treat IBD in clinical practice. Using combined heparin and sulfasalazine therapy, Gaffney and colleagues successfully treated 10 patients with ulcerative colitis poorly controlled on sulfasalazine and prednisolone[119]. Similarly, Evans *et al*. found that heparin was effective in treating corticosteroid-resistant ulcerative colitis[120], and Yoshikane *et al*. reported that heparin was very effective in the treatment of disseminated intravascular coagulation (DIC) caused by ulcerative colitis[121]. Recently, heparin was even suggested as a first line therapy in the treatment of severe colonic inflammatory bowel disease[122], but should be administered in hospitalized patients only because of the risk of possible serious bleeding[123]. To overcome bleeding side effect, low molecular weight heparin (LMWH) was employed as adjuvant therapy[124], and the patients showed good clinical and laboratory response with no severe adverse effects[125,126].

Apart from anticoagulation activity, the mechanisms by which heparin was able to treat IBD were considered to include its ability to inhibit the recruitment of neutrophils, reduce production of pro-inflammatory cytokines[127] and restore the high-affinity receptor binding of antiulcerogenic growth factor[128,129]. The ability of heparin to inhibit neutrophil activation, adhesion, and chemotaxis was also found in a mouse model of inflammatory bowel disorder[130,131], suggesting that balanced interactions between mast cells and neutrophils might be important for the development of IBD. In rat models of IBD, heparin revealed its ability to attenuate TNFα induced leucocyte rolling and CD11b dependent adhesion[132], reduce serum IL-6 level and improve microcirculatory disturbance in rectal walls[133,134]. Thus, preformed mast cell mediators seemed to have dual actions on the pathogenesis of IBD. On the one hand, tryptase, histamine, and TNF-α can cause damage in the intestinal wall, and on the other hand, heparin can protect the intestinal wall from damage.

INVOLVEMENT OF PGD2, LTC4 AND PAF IN PATHOGENESIS OF IBD

It was reported that mast cells in the actively involved areas of ulcerative colitis released greater amount of PGD2, in parallel to histamine and LTC4[135]. In a rat experimental colitis model, the time for stimulating PGD2 release was initiated within 1 h and increased 4 fold within 3 h[136]. This was accompanied by a significant granulocyte infiltration, indicating the likelihood of involvement of PGD2 in IBD. The basal release of LTC4 was enhanced in the gut of Crohn' s disease patients[137], but the meaning of this enhancement still remains uninvestigated.

It was found that mast cell activators, calcium ionophore A23187 and anti-IgE, were able to stimulate more PAF release from colon with ulcerative colitis than from normal colon, and this increased PAF release could be inhibited by steroids and 5-aminosalicylic acid[138,139]. The increased secretion of PAF was detected in the stool of patients with active Crohn' s disease, but not in that of patients with irritable bowel syndrome[140]. The level of PAF was also higher in colonic mucosa of patients with Crohn' s disease than in colonic mucosa of healthy controls[141]. These indicated that PAF might be involved in the pathogenesis of Crohn' s disease[142]. The elevated level of PAF in colon was likely to be the result of increased production by colonic epithelial cells[143], lamina propria mononuclear cells[144] and mast cells, and decreased PAF acetylhydrolase (the major PAF degradation enzyme activity)[145]. In patients with ulcerative colitis, colonic production of PAF was increased in comparison with control patients[146], and the level of PAF in the stool of patients with ulcerative colitis was much higher than that in the stool of healthy volunteers[147]. Since increased colonic production of PAF was correlated to local injury and inflammation[146], it implicats strongly that PAF is involved in the pathogenesis of ulcerative colitis. However, a randomized controlled trial with a specific PAF antagonist SR27417A showed that this compound had no significant effect on patients with active ulcerative colitis though it was safe in humans[148].

INVOLVEMENT OF CYTOKINES IN PATHOGENESIS OF IBD

Dozens of proinflammatory cytokines were reported to be involved in the pathogenesis of IBD, but only several of them have been considered to be therapeutic targets. TNFα was considered being secreted mainly from intestinal mast cells in IBD[78], and bacteria and anti-IgE were able to substantially enhance their release from mast cells[149]. The released mast cell products TNFα and histamine could then synergistically stimulate ion secretion from intestinal epithelium[150]. The anti-

TNFα therapy will be described below. In IBD, most intestinal mast cells produce IL-3, and this increased expression of IL-3 could be inhibited by administration of steroid[151]. IL-10, a cytokine which can be produced by human mast cells[152], was reported to have some anti-inflammatory role in IBD[153]. However, the results from clinical trials were heterogeneous[154]. IL-1 was found to be excessively released from patients with ulcerative colitis and could stimulate the short-circuit current response to IL-1[155]. As for IL-3, steroid could significantly inhibit IL-1 release in IBD[156]. Mast cells from healthy controls did not produce IL-5, but mast cells from patients with intestinal inflammatory disease could release a relatively large amount of IL-5[157]. However, the effect of IL-5 on IBD needs to be investigated. It is still in early days to understand the role of cytokines in IBD, therefore it is difficult to draw a conclusive line on the issue whether cytokine related therapy is beneficial for IBD.

HYPOTHESIS OF SELF-AMPLIFICATION MECHANISM OF MAST CELL DEGRANULATION IN GUT

Tryptase has been proved to be a unique marker of mast cell degranulation *in vitro* as it is more selective than histamine to mast cells. Inhibitors of tryptase[71,158] and chymase[159] have been discovered to possess the ability to inhibit histamine or tryptase release from human skin, tonsil, synovial[160] and colon mast cells[161,162], suggesting that they are likely to be developed as a novel class of mast cell stabilizers. Recently, a series of experiments with dispersed colon mast cells suggested there should be at least two pathways in man for mast cells to amplify their own activation-degranulation signals in an autocrine or paracrine manner, which may partially explain the phenomena that when a sensitized individual contacts allergen only once the local allergic response in the involved tissue or organ may last for days or weeks. These findings included both anti-IgE and calcium ionophore were able to induce significant release of tryptase and histamine from colon mast cells[163], histamine was a potent activator of human colon mast cells[164] and the agonists of PAR-2 and trypsin were potent secretagogues of human colon mast cells[165]. Since tryptase was reported to be able to activate human mast cells[71] and H_1 receptor antagonists terfenadine and cetirizine[166] were capable of inhibiting mast cell activation, the hypothesis of mast cell degranulation self-amplification mechanisms is that mast cell secretogogues induce mast cell degranulation, release histamine, then stimulate the adjacent mast cells or positively feedback to further stimulate its host mast cells through H_1 receptor, whereas released tryptase acts similarly to histamine through its receptor PAR-2 on mast cells (Figure 1).

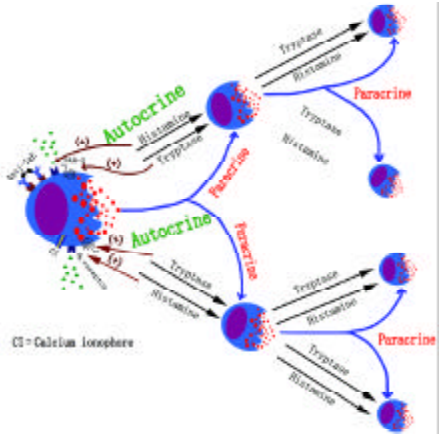

Figure 1 Hypothesis of self-amplification mechanism of mast cell degranulation in gut.

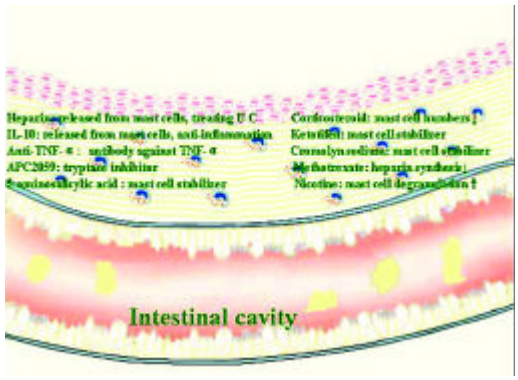

Figure 2 Association of mast cells with IBD therapies.

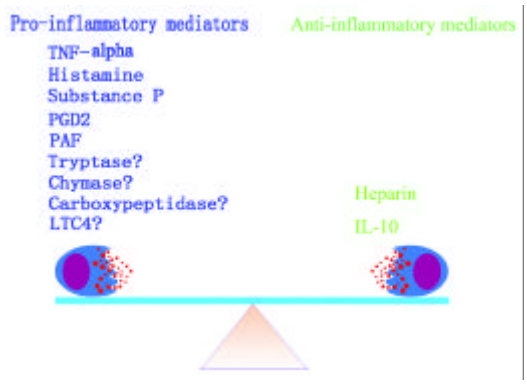

Figure 3 Balance between "pro-inflammatory" and "anti-inflammatory" mast cell mediators in IBD.

RELATIONSHIP BETWEEN THERAPIES FOR IBD AND MAST CELLS

Although aminosalicylates and corticosteroids remain the mainstream therapeutic drugs for the treatment of IBD[167], the mast cell related therapy should be given some close attentions. Besides heparin therapy mentioned above, anti-TNFα monoclonal antibody[168,169], infliximab in particular[170] showed promising results in treating Crohn' s disease. Thalidomide, an agent with antiangiogenic and immunomodulatory properties[171], possessed inhibitory activity towards TNFα[172] and was therapeutically effective in IBD[173]. Mast cell tryptase inhibitor APC2059 was also effective and safe in treating ulcerative colitis[174]. Even 5-aminosalicylic acid, an aminosalicylate drug was an effective inhibitor of anti-IgE induced histamine and PGD2 release from human intestinal mast cells[175]. Thus the beneficial effects of 5-aminosalicylic acid on IBD were at least partially due to its mast cell stabilizing activity. Similarly, the effective treatment of IBD by corticosteroids might also be partially associated with its action on mast cells as significantly reduced numbers of mast cells were observed in the colon throughout steroid therapy[176].

The ineffective treatment of ulcerative colitis by mast cell stabilizer, cromolyn sodium[177] was most likely due to the drug that did not affect release of histamine from colon mast cells[178]. However, it was recently found to be effective in treating chronic or recurrent enterocolitis in patients with Hirschsprung' s disease[179]. In 1992, Eliakim and colleagues found that ketotifen, a mast cell stabilizer, was able to significantly decrease mucosal damage of ulcerative colitis in an experimental colitis model[180] and inhibit accumulation of PGE2, ITB4 and LTC4 in ulcerative colitis colon mucosa organ-culture[181], suggesting that this anti-asthma drug may be useful for the treatment of IBD. Indeed, ketotifen was revealed to be effective in treating IBD with 5-aminosalicylate intolerance[182], and acute ulcerative colitis in

children[183], most likely through inhibition of mast cell and neutrophil degranulation[184].

It was surprising to learn that even immunomodulatory drug methotrexate, which showed promise in Crohn' s disease therapy[185] was able to inhibit heparin synthesis in mast cells[186], suggesting that the beneficial action of methotrexate on Crohn' s disease might be due to the reduction of heparin secretion from mast cells. Nicotine, an addictive component of tobacco, had a dual effect on IBD. It could ameliorate disease activity of ulcerative colitis but deteriorate disease process of Crohn' s disease[187]. Since nicotine was reported to be able to induce degranulation of mast cells[188], its dual action on IBD could be related to the locally imbalanced quantities of mast cell products, such as histamine and heparin. The association of these therapies with mast cells strongly indicates that mast cells are key cells in the development of IBD (Figure 2).

CONCLUSION

Mast cells are a key cell type, which is actively involved in the pathogenesis of IBD. The different actions of mast cell mediators in IBD suggest that there must be a balance between ' pro-IBD' and ' anti-IBD' mast cell mediators (Figure 3). Breaking this balance may cause diseases. There are at least two pathways, histamine pathway and tryptase pathway, for mast cells to amplify their degranulation signals. Each of them is likely to act in either autocrine or paracrine manner. These mast cell degranulation signal amplification mechanisms may be the key event in the pathophysiological process of long-lasting local response of mast cell associated diseases such as IBD, asthma and rhinitis.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Induction of tryptase and histamine release from human colon mast cells by IgE dependent or independent mechanisms

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Abstract

AIM: To investigate the tryptase and histamine release ability of human colon mast cells upon IgE dependent or independent activation and the potential mechanisms.

METHODS: Enzymatically dispersed cells from human colons were challenged with anti-IgE or calcium ionophore A23187, and the cell supernatants after challenge were collected. Both concentration dependent and time course studies with anti-IgE or calcium ionophore A23187 were performed. Tryptase release was determined with a sandwich ELISA procedure and histamine release was measured using a glass fibre-based fluorometric assay.

RESULTS: Both anti-IgE and calcium ionophore were able to induce dose dependent release of histamine from colon mast cells with up to approximately 60% and 25% net histamine release being achieved with 1 μg/mL calcium ionophore and 10 μg/mL anti-IgE, respectively. Dose dependent release of tryptase was also observed with up to approximately 19 ng/mL and 21 ng/mL release of tryptase being achieved with 10 μg/mL anti-IgE and 1 μg/mL calcium ionophore, respectively. Time course study revealed that both tryptase and histamine release from colon mast cells stimulated by anti-IgE initiated within 10 sec and reached their maximum release at 6 min following challenge. Pretreatment of cells with metabolic inhibitors abolished the actions of anti-IgE as well as calcium ionophore. Tryptase and histamine release, particularly that induced by calcium ionophore was inhibited by pretreatment of cells with pertussis toxin.

CONCLUSION: Both anti-IgE and calcium ionophore are able to induce significant release of tryptase and histamine from colon mast cells, indicating that this cell type is likely to contribute to the pathogenesis of colitis and other mast cell associated intestinal diseases.

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INTRODUCTION

Increased numbers of mast cells have been found in the epithelium of intestine of the patients with ulcerative colitis and Crohn' s disease[1,2]. Through releasing its proinflammatory mediators including tryptase, histamine, heparin, and other preformed or newly synthesized mast cell products[3], mast cells actively participate in the pathogenesis of inflammatory bowel diseases[4].

Tryptase is a tetrameric serine proteinase that constitutes some 20% of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells[5] in a catalytically active form[6]. Upon degranulation, tryptase is released from mast cells along with chymase, histamine, and other mast cell products. In recent years, evidence has been emerging that this major secretory product of human mast cells may be a key mediator of allergic inflammation and a promising target for therapeutic intervention[3] as it has been found to be able to induce microvascular leakage in the skin of guinea pig[7], bronchoconstriction[8] in allergic sheep airways, inflammatory cell accumulation in peritoneum of mouse[9] and release of IL-8 from epithelial cells[10].

For more than four decades, histamine has been widely used as a marker of mast cell degranulation *in vitro*, and numerous anti-allergic drugs such as sodium cromoglycate, lodoxamide, salbutamal, ketotifen, terfenadine and cetirizine[11,12] and salmeteral[13] were reported to be able to inhibit anti-IgE induced histamine release from human tonsil, skin or lung mast cells. However, little is known of the properties of tryptase release from human mast cells, and even histamine release properties of human colon mast cells were relatively less studied. We therefore parallelly investigated the tryptase and histamine release properties of human colon mast cells by using the same preparations of challenged mast cells in the current study.

MATERIALS AND METHODS

Reagents

The following compounds were purchased from Sigma (St. Louis, USA): collagenase (type I), hyaluronidase (type I), bovine serum albumin (BSA, fraction V), penicillin and streptomycin, calcium ionophore A23187, antimycin A, 2-deoxy-D-glucose, pertussis toxin. Goat anti-human IgE (inactivated) was from Serotec (Kidlington, Oxford, UK). FCS and minimum essential medium (MEM) containing 25 mM *N*-2-hydroxylethylpiperazine-*N*' -2-ethane sulphonic acid (HEPES) were from Gibco (Paisley, Renfrewshire, UK). A polyclonal antibody and a monoclonal antibody against tryptase were donated by Dr Andrew F. Walls (University of Southampton, UK). Histamine plate was from RefLab (Copenhagen, Denmark). HEPES and all other chemicals were of analytical grade.

Dispersion of mast cells

Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removing fat, tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm^3, and then incubated with 1.5 mg/ml collagenase and 0.75 mg/ml

hyaluronidase in MEM containing 2% fetal calf serum (1 g colon/10 ml buffer) for 70 min at 37 ℃. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100 μm diameter), washed and maintained in MEM (containing 10% FCS, 200 U/ml penicillin, 200 μg/ml streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after stained by alcine blue, ranged from 3.5% to 5.4%.

Mast cell challenge

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with $CaCl_2$ and $MgCl_2$ (complete HBSS), and 100 μl aliquots containing 4-6×10^3 mast cells were added to a 50 μl anti-IgE, calcium ionophore or control buffer alone and incubated for 15 min at 37 ℃. The reaction was terminated by the addition of 150 μl ice cold incomplete HBSS and the tubes were centrifuged immediately (500 g, 10 min, 4 ℃). All experiments were performed in duplicate. For the experiments with pertussis toxin, cells were incubated with 0.1 or 1.0 μg/ml pertussis toxin for four hours at 37 ℃, and then washed with HBSS before adding stimulus. Similarly, for the experiments with metabolic inhibitors, cells were incubated with 2-deoxy-D-glucose (10 mM) and antimycin A (1 μM) for 40 min at 37 ℃ before challenged with stimulus. For the measurement of total histamine concentration, in certain tubes the suspension was boiled for 6 min. Supernatants were stored at -20 ℃ until use.

Tryptase and histamine measurement

Tryptase concentrations were measured with a sandwich ELISA procedure with a specific polyclonal antibody against human tryptase as the capture antibody and AA5 a monoclonal antibody specific for human tryptase as the detecting antibody[14]. Histamine concentrations were determined using a glass fibre-based fluorometric assay[15].

Statistical analysis

Data are shown as mean±SEM for the number of experiments (n) indicated, and the paired Student' s t test was applied to evaluate two independent samples. In all analyses $P<0.05$ was taken as statistically significant.

RESULTS

Effect of anti-IgE and calcium ionophore on tryptase and histamine release from colon mast cells

Both anti-IgE and calcium ionophore were able to induce a dose dependent release of histamine from colon mast cells with up to approximately 60% and 25% net histamine release being achieved with 1 μg/mL calcium ionophore and 10 μg/mL anti-IgE, respectively. Increasing the concentrations of calcium ionophore up to 10 μg/mL and anti-IgE up to 100 μg/mL failed to provoke any further release of histamine from colon mast cells (Figure 1). Dose dependent release of tryptase was also observed when dispersed colon mast cells were incubated with calcium ionophore or anti-IgE. Up to approximately 19 ng/mL and 21 ng/mL release of tryptase were achieved with 10 μg/mL anti-IgE and 1 μg/mL calcium ionophore, respectively. Only as little as 0.1 μg/mL calcium ionophore and 1 μg/mL anti-IgE were required to elicit a significant release of tryptase. Similar to histamine release, increasing the concentrations of calcium ionophore to more than 1 μg/mL and anti-IgE more than 10 μg/mL did not stimulate more tryptase release from colon mast cells (Figure 2). There was a significant correlation between the quantities of histamine and tryptase released in response to anti-IgE (Pearson correlation: r=0.939, P=0.005) and calcium ionophore (Pearson correlation: r=0.973, P=0.001).

Time course study revealed that both tryptase and histamine release from colon mast cells stimulated by anti-IgE initiated within 10 sec when cells were incubated with the stimulus, the release was then steadily increased while incubation periods were prolonged. Tryptase and histamine reached their maximum release at 6 min following incubation. Prolonging the incubation period from 6 min to 15 min had little effect on the release of tryptase and histamine (Figure 3). The time course pattern of tryptase release provoked by calcium ionophore was similar to that induced by anti-IgE with approximately 45% tryptase released within 10 sec. This was different from the time course for calcium ionophore induced histamine release, which showed as much as approximately 70% of histamine released within 10 sec of challenge (Figure 4).

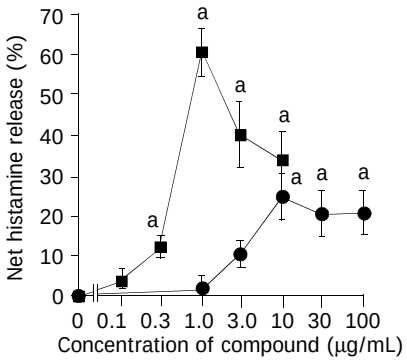

Figure 1 Anti-IgE and calcium ionophore induced histamine release from colon mast cells. The values shown are mean±SEM for four separate experiments. Stimulus or HBSS alone was incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with spontaneous release group (paired Student's t test).

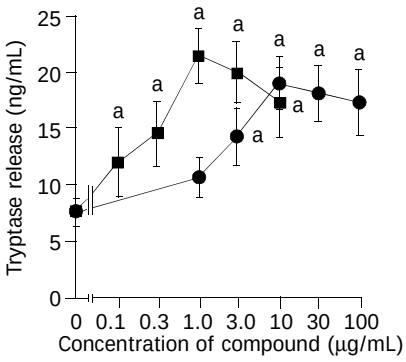

Figure 2 Anti-IgE and calcium ionophore induced tryptase release from colon mast cells. The values shown are mean±SEM for four separate experiments. Stimulus or HBSS alone was incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with spontaneous release group (paired Student's t test).

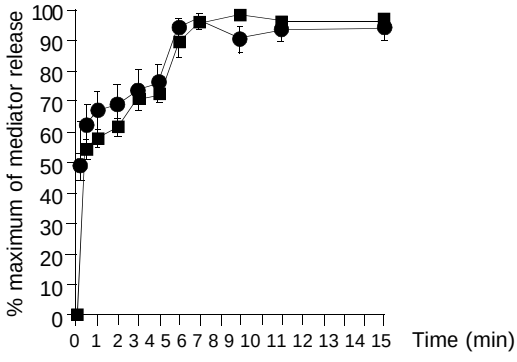

Figure 3 Time course for anti-IgE (10 μg / ml) induced release of tryptase (—■—) and histamine (—●—) from colon mast cells. Data shown are mean±SEM of four separate experiments.

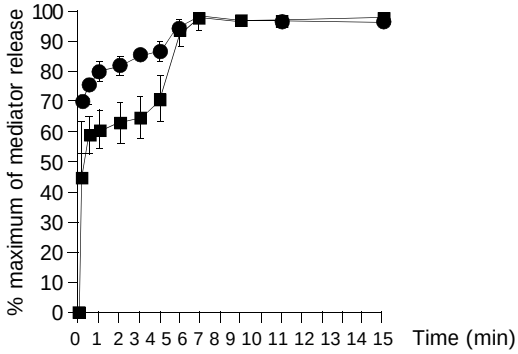

Figure 4 Time course for calcium ionophore (1 μg / ml) induced release of tryptase (—■—) and histamine (—●—) from colon mast cells. Data shown are mean±SEM of four separate experiments.

Inhibition of anti-IgE and calcium ionophore induced tryptase and histamine release by pertussis toxin and metabolic inhibitors

Pretreatment of cells with metabolic inhibitors abolished the actions of anti-IgE as well as calcium ionophore. Tryptase and histamine release, particularly that induced by calcium ionophore was inhibited by pretreatment of cells with pertussis toxin (Table 1, Table 2).

Table 1 Effects of pertussis toxin and metabolic inhibitors on anti-IgE induced histamine and tryptase release from colonic mast cells

Treatment		Net mediator release (ng / mL)	
		Tryptase	Histamine
Untreated		11.4±2.7	14.0±3.2
Pertussis toxin	0.1 μg / mL	3.2±1.3[a]	5.4±1.7[a]
	1.0 μg / mL	4.0±1.6[a]	4.0±1.9[a]
Metabolic inhibitors		-2.1±1.7[a]	-2.6±1.7[a]

The values shown are mean±SEM of four separate experiments performed in duplicate. [a]$P<0.05$ compared with the untreated group (paired Student's *t* test). Metabolic inhibitors=1 μM antimycin A+10 mM 2-deoxy-D-glucose.

Table 2 Effects of pertussis toxin and metabolic inhibitors on calcium ionophore induced tryptase and histamine release from colonic mast cells

Treatment		Mediator release (ng / mL)	
		Tryptase	Histamine
Untreated		13.8±2.4	34.3±3.4
Pertussis toxin	0.1 μg / mL	4.0±1.6[a]	2.7±2.8[a]
	1.0 μg / mL	1.4±1.8[a]	-0.1±1.3[a]
Metabolic inhibitors		2.0±1.4[a]	1.8±2.6[a]

The values shown are mean±SEM of four separate experiments performed in duplicate. [a]$P<0.05$ compared with the untreated group (paired Student's *t* test). Metabolic inhibitors=1 μM antimycin A+10 mM 2-deoxy-$_D$-glucose.

DISCUSSION

We have reported for the first time the *in vitro* tryptase release ability of human colon mast cells. This is particularly important when tryptase and histamine, the two major mast cell mediators were investigated in the same experiments, which would not only prove that the two mediators were released from mast cells unnecessarily at a constantly parallel ratio, but also revealed that histamine was released slightly faster than tryptase upon degranulation.

Approximately 25% histamine and 10% tryptase (presumably 10 pg tryptase per mast cell[16], and 5000 cells in each test tube) were released from colon mast cells in response to IgE dependent provocation, indicating that only a proportion of mast cells was activated or the granule contents in activated mast cells were partially released. The maximum release of histamine and tryptase was induced by 10 μg/mL rather than higher concentrations of anti-IgE, suggesting that colon mast cells would give their maximum response to a sufficient stimulation, but not an excess stimulation, which showed a self-protection mechanism of colon mast cells.

Approximately 45% degranulation occurred within 10 sec of IgE dependent stimulation, suggesting that colon mast cells were able to quickly respond to allergen challenge, but they required about 6 min to reach their full capacity (maximum histamine or tryptase release). The time course also revealed that histamine was released slightly faster than tryptase upon degranulation, and this was particularly evident with calcium ionophore. The peak histamine release induced by anti-IgE and calcium ionophore occurred at 6 min following stimulation of colon mast cells, and appeared much faster than the occurrence of peak histamine release with lung mast cells, which required at least 15 min to reach its peak following anti-IgE and calcium ionophore stimulation[17].

Pretreatment of cells with metabolic inhibitors antimycin A, which blocks the oxidative phosphorylation process of cells, and 2-deoxy-D-glucose which blocks anaerobic metabolism pathway in cells, abolished the actions of anti-IgE as well as calcium ionophore, indicating that the induced release of these two mediators was a non-cytotoxic process, and was dependent on cell energy supply. Tryptase and histamine release, particularly that induced by calcium ionophore was inhibited by pretreatment of cells with pertussis toxin, suggesting that the degranulation process elicited by anti-IgE and calcium ionophore was associated with activation of G-protein coupled receptors.

Over the last decade, a number of functions of mast cell tryptase have been discovered. However, the mechanism by which tryptase acts on cells is still unclear. The finding that proteinase-activated receptor-2 (PAR-2) on cos cells could be a receptor for tryptase[18] suggested that the effects of tryptase on various types of cells were associated with this type of receptor. Since both tryptase[7,15] and PAR-2 agonists[19] were able to activate human mast cells, it is likely that there is a self-amplification mechanism of mast cell degranulation, which would contribute largely to the pathogenesis of mast cell related diseases including inflammatory bowel diseases. The report that induction of intestinal inflammation in mouse by activation of PAR-2[20] and PAR-2 as an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis[21] suggested the potential roles of PAR-2 in inflammatory bowel diseases.

Histamine has long been accepted as a specific marker of mast cell degranulation. Tryptase however, is seldom used in the same way though it has been employed as a specific marker of mast cells since 1980's. Based on the results in the current study and the evidence that these two mediators are located in the same secretory granules of mast cells, we believe that human tryptase can serve as an indicator of mast cell degranulation *in vitro*. In conclusion, both anti-IgE and calcium ionophore are able to induce a significant release of tryptase and histamine from colon mast cells, indicating that this cell type is likely to contribute to the pathogenesis of colitis and other mast cell associated intestinal diseases.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Modulation of tryptase secretion from human colon mast cells by histamine

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Abstract

AIM: To investigate the ability of histamine to modulate tryptase release from human colon mast cells and the potential mechanisms.

METHODS: Enzymatically dispersed cells from human colons were challenged with histamine, anti-IgE or calcium ionophore A23187 (CI), and the cell supernatants after challenge were collected. Tryptase release was determined with a sandwich ELISA procedure.

RESULTS: Histamine at concentrations from 1 ng/mL was able to induce a "bell" shape dose related release of tryptase from colon mast cells. The maximum release of tryptase was approximately 3.5 fold more than spontaneous release. As little as 10 ng/mL histamine showed a similar potency to 10 μg/mL anti-IgE in induction of tryptase release. Histamine induced release of tryptase initiated at 10 s when histamine (100 ng/mL) was added to cells, gradually increased thereafter, and completed at 5 min. Both pertussis toxin or metabolic inhibitors were able to inhibit histamine induced tryptase release. When histamine and anti-IgE were added to colon mast cells at the same time, the quantity of tryptase released was similar to that induced by anti-IgE alone. The similar results were observed with CI. However, when various concentrations of histamine were incubated with cells for 20 min before adding anti-IgE or CI, the quantity of tryptase released was similar to that was induced by histamine alone.

CONCLUSION: Histamine is a potent activator of human colon mast cells, which represents a novel and pivotal self-amplification mechanism of mast cell degranulation.

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INTRODUCTION

It has been reported that mast cells and their inflammatory mediators are closely associated to a number of intestinal diseases including idiopathic inflammatory bowel disease[1], chronic ulcerative colitis[2], Crohn's disease[3] and collagenous colitis[4]. Through release their proinflammatory mediators including histamine, tryptase, chymase, heparin and some cytokines[5], mast cells actively participate in the pathogenesis of these intestinal diseases.

Tryptase is a tetrameric serine proteinase that constitutes some 20% of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells[6] in a catalytically active form[7]. Relatively higher secretion of tryptase has been detected in ulcerative colitis[8], implicating that this mediator is involved in the pathogenesis of intestinal diseases. Evidence is emerging that tryptase may be a key mediator of allergic inflammation and a promising target for therapeutic intervention[9] as it has been found to be able to induce microvascular leakage in the skin of guinea pig[10], bronchoconstriction[11] in allergic sheep airways, inflammatory cell accumulation in peritoneum of mouse[12] and release of IL-8 from epithelial cells[13]. Moreover, tryptase has long been recognised as a marker of mast cells[14,15], and an indicator of mast cell degranulation *in vivo*[16,17]. However, the application of tryptase as a unique marker of mast cell degranulation in mast cell challenge study *in vitro* was only started recently[18]. This was largely due to the lack of adequate assay to detect this mast cell product.

Histamine, on the other hand, has been widely employed as a marker of mast cell degranulation in mast cell challenge studies over the last four decades. But as a activator of mast cells it has hardly been examined. Since increased levels of histamine or enhanced histamine metabolism have been observed in collagenous colitis, food allergy[19], Crohn's disease[20], ulcerative colitis[20,21] and allergic enteropathy[21], this proinflammatory mediator is likely to participate in the pathogenesis of these diseases. In the current study, we investigated the potential of histamine to activate human colon mast cells *in vitro* in order to understand further the role of histamine in inflammatory bowel diseases.

MATERIALS AND METHODS

Reagents

The following compounds were purchased from Sigma (St. Louis, Mo., USA): CI, histamine dihydrochloride, collagenase (type I), hyaluronidase (type I), antimycin A, 2-deoxy-D-glucose, pertussis toxin, bovine serum albumin (BSA, fraction V), penicillin and streptomycin, extravidin peroxidase, *o*-phenylene diamine, biotin conjugate sheep anti-mouse immunoglobulins, extr-avidin peroxidase. Minimum essential medium (MEM) containing 25 mM *N*-2-hydroxylethylpiperazine-*N*' -2-ethane sulphonic acid (HEPES) and FCS was from Gibco (Paisley, Renfrewshire, UK). Goat anti-human IgE (inactivated) was from Serotec (Kidlington, Oxford, UK). A polyclonal antibody and a monoclonal antibody against tryptase were donated by Dr. Andrew F. Walls (University of Southampton, UK). HEPES and all other chemicals were of analytical grade.

Dispersion of mast cells

The mast cell dispersion procedure was similar to that described

previously[21,22]. Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removal of fat, the tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm^3, and then incubated with 1.5 mg/mL collagenase and 0.75 mg/mL hyaluronidase in MEM containing 2% fetal calf serum (1 g colon/10 mL buffer) for 70 min at 37 ℃. Dispersed cells were separated from the undigested tissue by filtration through a nylon gauze (pore size 100 μm diameter), washed and maintained in MEM (containing 10% FCS, 200 U/mL penicillin, 200 μg/mL streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after staining by Alcine blue, ranged from 3.6% to 5.8%.

Mast cell challenge

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (complete HBSS), and 100 μL aliquots containing 4-6×10^3 mast cells was added to 50 μL histamine, anti-IgE, CI or buffer alone and incubated for 15 min at 37 ℃. The reaction was terminated by the addition of 150 μL ice cold incomplete HBSS and the tubes were centrifuged immediately (500 g, 10 min, 4 ℃). All experiments were performed in duplicate. For the experiments with pertussis toxin, cells were incubated with 0.1 or 1.0 μg/mL pertussis toxin for four hours at 37 ℃, and then washed with HBSS before adding stimulus. Similarly, for the experiments with metabolic inhibitors, cells were incubated with 2-deoxy-$_D$-glucose (10 mmol/L) and antimycin A (1 μmol/L) for 40 min at 37 ℃ before challenged with stimulus. Supernatants were stored at -20 ℃ until use. As reported previously that 10 μg/mL anti-IgE and 1 μg/mL CI were the optimal concentrations for the induction of tryptase released from human colon mast cells[18], therefore they were selected as the standard concentrations throughout the study.

Tryptase measurement

Tryptase concentrations were measured using a sandwich ELISA procedure with a specific polyclonal antibody against human tryptase as the capture antibody and AA5 a monoclonal antibody specific for human tryptase as the detecting antibody[23].

Statistical analysis

Data were shown as mean±SEM for the number of experiments (n) indicated, and the paired Student' s t test was applied to evaluate two independent samples. In all analyses, $P<0.05$ was taken as statistically significant.

RESULTS

Induction of tryptase release by histamine

Histamine at the concentration of 1 ng/mL was able to induce a ' bell' shape dose related release of tryptase from colon mast cells. The maximal release of tryptase was approximately 3.5 fold more than spontaneous release provoked by 100 ng/mL histamine. Relatively less tryptase was released when 1 000 ng/mL and 10 000 ng/mL histamine were incubated with mast cells. As little as 10 ng/mL histamine showed a similar potency to 10 μg/mL anti-IgE in induction of tryptase release from colon mast cells (Figure 1).

Time course for histamine induced tryptase release

Histamine induced release of tryptase initiated at 10 s when histamine (100 ng/mL) was added to cells, gradually increased thereafter, and completed at 5 min (Figure 2).

Effects of pertussis toxin and metabolic inhibitors on histamine induced tryptase release

Tryptase release induced by histamine was reduced to the baseline level by pretreatment of colon mast cells with pertussis toxin or metabolic inhibitors. The same treatment was also able to slightly decrease the spontaneous tryptase release from mast cells (Table 1).

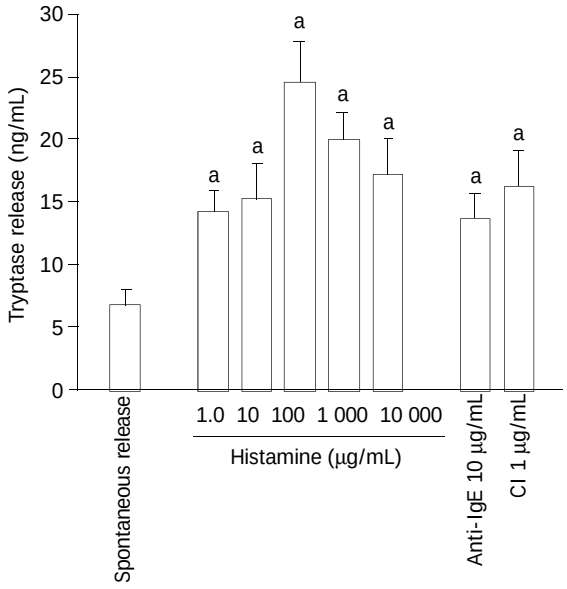

Figure 1 Histamine, anti-IgE and calcium ionophore (CI) induced tryptase release from colon mast cells. The values shown are mean±SEM for six separate experiments. Stimulus or HBSS alone was incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with spontaneous release group (paired Student's t test).

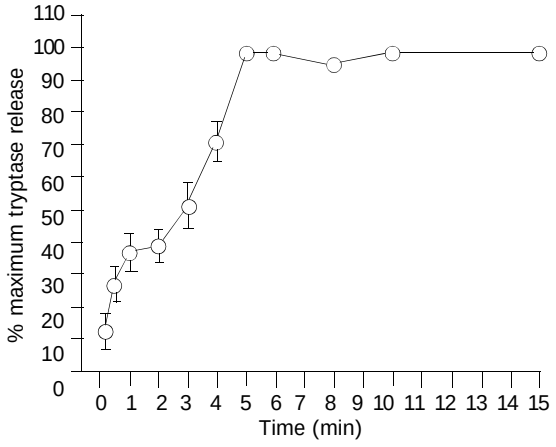

Figure 2 Time course for histamine (100 ng/mL) induced release of tryptase from colon mast cells. Data shown are mean±SEM of five separate experiments.

Table 1 Effects of pertussis toxin (1.0 μg/mL) and metabolic inhibitors on histamine induced tryptase release from colon mast cells

Treatment	Tryptase release (ng/mL)		
	Hist 100 μg/mL	Hist 1 000 μg/mL	Buffer alone
Untreated	32±5.5	30.5±3.9	20.5±2.8
Pertussis toxin	17.1±1.8[a]	16.7±1.7[a]	17.6±2.7
Metabolic inhibitors	18.8±3.5[a]	17.5±1.9[a]	16.3±1.9

The values shown are mean±SEM of five separate experiments performed in duplicate. [a]$P<0.05$ compared with the untreated group (paired Student's t test). Metabolic inhibitors=1 μM antimycin A+10 mM 2-deoxy-$_D$-glucose. Hist=histamine.

Effect of histamine on anti-IgE and CI induced tryptase release

When 100 ng/mL or 1 000 ng/mL histamine and anti-IgE were added to colon mast cells at the same time, tryptase release was significantly less than that induced by addition of the corresponding concentration of histamine alone. The similar results were observed when the same concentrations of histamine were added to cells at the same time with CI, though there was no significant difference between histamine alone and histamine plus CI (Figure 3). However, addition of various concentrations of histamine to colon mast cells 20 min before placing anti-IgE or CI, tryptase release was similar to that induced by the corresponding histamine alone, except for 10 ng/mL histamine with CI, indicating a synergistic action between them (Figure 4).

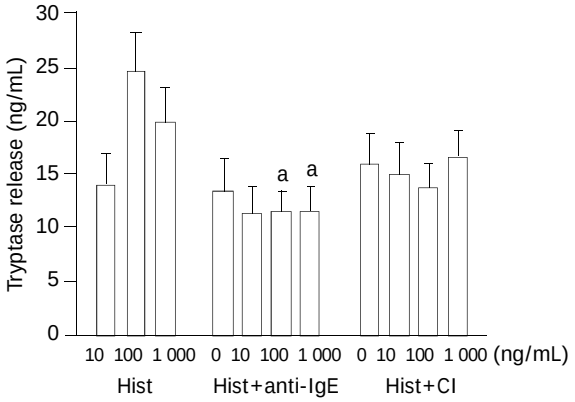

Figure 3 Effect of co-addition of histamine (hist) and anti-IgE or calcium ionophore (CI) on tryptase release from colon mast cells. The values shown are mean±SEM for six separate experiments. Various concentrations of histamine and anti-IgE or CI were added to cells at the same time, and then incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with the corresponding histamine alone group (paired Student's *t* test).

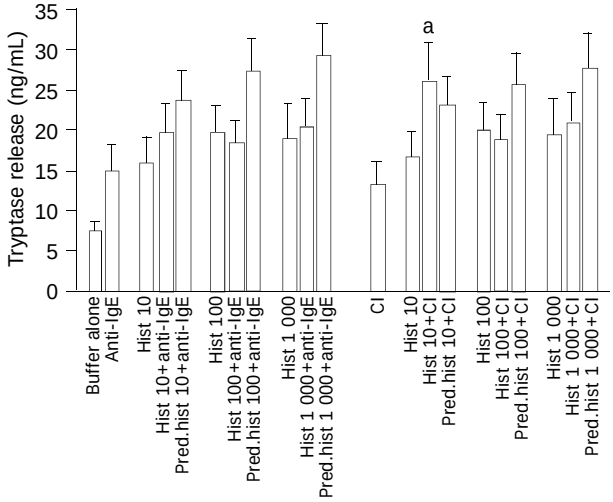

Figure 4 Effect of histamine (hist) on anti-IgE or calcium ionophore (CI) induced tryptase release from colon mast cells. The values shown are mean±SEM for six separate experiments. Various concentrations of histamine were incubated with cells for 20 min before addition of anti-IgE or CI. [a]$P<0.05$ compared with the corresponding histamine alone group (paired Student's *t* test). Pred.hist + anti-IgE or CI=the sum of tryptase release induced by histamine alone and anti-IgE or CI alone.

DISCUSSION

The finding that histamine is a potent activator of human colon mast cells demonstrated a novel and pivotal self-amplification mechanism of human mast cell degranulation, that is mast cells release histamine upon degranulation, and then the released histamine activates the adjacent mast cells. Thus, there are at least two self-amplification mechanisms in human mast cells upon degranulation, including tryptase and PAR-2 feedback process previously reported[22,23] and histamine inducing tryptase release.

Histamine appeared a potent secretogogue of tryptase, as little as 10 ng/mL histamine could share a similar potency of 10 μg/mL anti-IgE. This was a surprising result, but it clearly demonstrate a novel self-amplification mechanism of mast cell degranulation as the concentration of histamine inside the mast cell granules was estimated over 100 m mol/mL[24]. Interestingly, histamine concentration at 1 000 ng/mL or above was able to induce less tryptase release from mast cells than 100 ng/mL, which may represent a novel self-protection mechanism of mast cells. Histamine induced tryptase release started slower than that elicited by anti-IgE, CI[18] or tc- LIGRLO[23], but completed 1 min earlier than that induced by anti-IgE and CI, indicating that it has its own activation-degranulation passway in mast cells. Pretreatment of cells with metabolic inhibitors antimycin A which blocks the oxidative phosphorylation process of cells, and 2-deoxy-D-glucose which blocks anaerobic metabolism pathway in cells, abolished the action of histamine, indicating that histamine induced release of tryptase is a non-cytotoxic process depending on cell energy supply. Tryptase release provoked by histamine was also inhibited by pretreatment of cells with pertussis toxin, suggesting that the degranulation process is associated with the activation of G-protein coupled receptors[25].

When histamine and anti-IgE were placed to mast cells at the same time, the quantity of released tryptase was similar to that elicited by anti-IgE alone, much less than that provoked by histamine alone, implicating that tryptase released from colon mast cells was mainly induced by anti-IgE, rather than by histamine. The reason for this was that the initiation of anti-IgE induced tryptase release was quicker than that induced by histamine, and mast cells only accepted one type of stimulation at a time[26,27]. Similar phenomena were observed with CI, and CI provoked mast cell degranulation apparently faster than that induced by histamine. In contrast, addition of various concentrations of histamine to colon mast cells 20 min before placing anti-IgE or CI, tryptase release was similar to that induced by the corresponding histamine alone, which proved further that colon mast cells were only able to respond to one optimal activation at a time, and the desensitized mast cells did not respond to any further stimulation. This behavior is an effective self-protection mechanism of mast cells. However, a synergistic action between 10 ng/mL histamine and CI, but not anti-IgE was observed, suggesting that mast cells experiencing a non-optimal stimulation may still have ability to respond to a further stimulation. In conclusion, histamine is a potent activator of human colon mast cells, and represents a novel and pivotal self-amplification mechanism of mast cell degranulation.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Activation of human colon mast cells through proteinase activated receptor-2

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Abstract

AIM: To investigate the ability of agonists of PAR-2 to stimulate release of tryptase and histamine from human colon mast cells and the potential mechanisms.

METHODS: Enzymatically dispersed cells from human colons were challenged with tc-LIGRLO, tc-OLRGIL, SLIGKV, VKGILS, trypsin, anti-IgE or calcium ionophore A23187, and the cell supernatants after challenge were collected. Tryptase release was determined with a sandwich ELISA procedure and histamine release was measured using a glass fibre-based fluorometric assay.

RESULTS: Both PAR-2 agonists tc-LIGRLO-NH_2 and SLIGKV-NH_2 were able to induce dose dependent release of tryptase and histamine from colon mast cells. More than 2.5 fold increase in both tryptase and histamine release was provoked by 100 μmol/mL tc-LIGRLO-NH_2, in comparison with only 2.0 fold increase being stimulated by SLIGKV-NH_2. The reverse peptides tc-OLRGIL-NH_2 and VKGILS –NH_2 at the concentrations tested had no effect on the release of these two mediators. The maximum tryptase release elicited by tc-LIGRLO-NH_2 was similar to that induced by anti-IgE (10 μg/mL) or calcium ionophore (1 μg/mL), though the latter was a more potent stimulus for histamine release. Both histamine and tryptase release in response to tc-LIGRLO-NH_2 were completed within 3 min. Trypsin at concentrations from 1.0 to 100 μg/mL was capable of provoking a dose dependent release of tryptase as well as histamine with a maximum of 16 ng/mL tryptase and 14 ng/mL histamine release being achieved. An approximately 80% and 70% inhibition of trypsin induced release of tryptase and histamine were observed with SBTI, respectively. Pretreatment of cells with metabolic inhibitors or pertussis toxin abolished the actions of tc-LIGRLO-NH_2, SLIGKV-NH_2 and trypsin.

CONCLUSION: The agonists of PAR-2 and trypsin are potent secretagogues of human colon mast cells, which are likely to contribute to the development of inflammatory disorders in human gut.

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INTRODUCTION

PAR-2 is a 7-transmembrane G protein-coupled receptor, which can be activated by serine proteinase trypsin and mast cell tryptase[1], and some synthetic peptides corresponding to the N-terminal tethered ligand sequences that are unmasked by proteolytic cleavage including SLIGKV in man[2] and SLIGRL in rodent[3].

In gastrointestinal tract, PAR-2 activation has been found to be actively involved in a number of inflammatory processes including induction of granulocyte infiltration, colon edema, tissue damage in mouse[4,5], induction of gastric mucus secretion and mucosal cytoprotection[6] in rat, excitation of submucosal neurons of guinea pig small intestine[7,8] and activation of ion secretion from human colon[9]. Moreover, upregulation of expression of PAR-2 in ulcerative colitis[10] suggested a strong possibility of involvement of mast cell tryptase and trypsin in inflammatory bowel disease.

Indeed, increased numbers of mast cells were observed in chronic ulcerative colitis[11] and Crohn' s disease[12], elevated histamine levels or enhanced histamine metabolism were found in collagenous colitis, food allergy[13], Crohn' s disease[14], ulcerative colitis[14,15] and allergic enteropathy[15] and increased levels of tryptase were detected in ulcerative colitis[16], indicating that these two proinflammatory mediators are involved in the pathogenesis of certain gastrointestinal diseases.

We have reported previously that mast cell tryptase was able to activate mast cells[17,18], which presents a self-amplification mechanism of mast cell degranulation. Since the receptor of tryptase, PAR-2 was localized on human mast cells[19] and PAR-2 agonists were reported to be capable of activating rat peritoneal mast cells[20], it is likely that PAR-2 agonists and trypsin may have ability to activate human mast cells. We therefore examined the effect of trypsin and PAR-2 agonists on tryptase and histamine release from human colon mast cells in the current study.

MATERIALS AND METHODS

Reagents

The following compounds were purchased from Sigma (St. Louis, USA): collagenase (type I), hyaluronidase (type I), soy bean trypsin inhibitor (SBTI), bovine serum albumin (BSA, fraction V), penicillin and streptomycin, calcium ionophore A23187, antimycin A, human trypsin, 2-deoxy-D-glucose, pertussis toxin. Goat anti-human IgE (inactivated) was from Serotec (Kidlington, Oxford, UK). FCS and minimum essential medium (MEM) containing 25 mM *N*-2-hydroxylethylpiperazine-*N*' -2 -ethane sulphonic acid (HEPES) were from Gibco (Paisley, Renfrewshire, UK). Peptides SLIGKV-NH_2, VKGILS-NH_2, trans-cinnamoyl-Leu-Ile-Gly-Arg-Leu- Orn-amide (tc-LIGRLO) and trans-cinnamoyl-Orn-Leu- Arg-Gly-Ile-Leu-amide

(tc-OLRGIL) were >98% purity and from Meilian Corporation (Xian, China). A polyclonal antibody and a monoclonal antibody against tryptase were donated by Dr Andrew F. Walls (University of Southampton, UK). Histamine plate was from RefLab (Copenhagen, Denmark). HEPES and all other chemicals were of analytical grade.

Dispersion of mast cells

The mast cell dispersion procedure was similar to the one described previously[21,22]. Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removal of fat, tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm^3, and then incubated with 1.5 mg/mL collagenase and 0.75 mg/mL hyaluronidase in MEM containing 2% fetal calf serum (1 g colon/10 mL buffer) for 70 min at 37 ℃. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100 μm diameter), washed and maintained in MEM (containing 10% FCS, 200 U/mL penicillin, 200 μg/mL streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after stained by alcine blue, ranged from 3.5% to 5.5%.

Mast cell challenge

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (complete HBSS), and 100 μL aliquots containing $4\text{-}6\times10^3$ mast cells were added to a 50 μL tc-LIGRLO, tc-OLRGIL, SLIGKV, VKGILS, trypsin, control secretagogue or buffer alone and incubated for 15 min at 37 ℃. The reaction was terminated by the addition of 150 μL ice cold incomplete HBSS and the tubes were centrifuged immediately (500 g, 10 min, 4 ℃). All experiments were performed in duplicate. For the experiments with pertussis toxin, cells were incubated with 0.1 or 1.0 μg/mL pertussis toxin for four hours at 37 ℃, and then washed with HBSS before adding stimulus. Similarly, for the experiments with metabolic inhibitors, cells were incubated with 2-deoxy-D-glucose (10 mmol/L) and antimycin A (1 μmol/L) for 40 min at 37 ℃ before challenged with stimulus. For the measurement of total histamine concentration in certain tubes, the suspension was boiled for 6 min. Supernatants were stored at -20 ℃ until use.

Tryptase and histamine measurement

Tryptase concentrations were measured with a sandwich ELISA procedure with a specific polyclonal antibody against human tryptase as the capture antibody and AA5 a monoclonal antibody specific for human tryptase as the detecting antibody[23]. Histamine concentrations were determined using a glass fibre-based fluorometric assay[17].

Statistical analysis

Data are shown as mean±SEM for the number of experiments (*n*) indicated, and the paired Student' s *t* test was applied to evaluate two independent samples. In all analyses $P<0.05$ was taken as statistically significant.

RESULTS

Induction of tryptase release by agonists of PAR-2

Both PAR-2 agonists tc-LIGRLO-NH_2 and SLIGKV-NH_2 were able to induce dose dependent release of tryptase from colon mast cells (Figure 1). More than 2.5 fold increase in tryptase release was provoked by 100 μmol/mL tc-LIGRLO-NH_2, even at a concentration as low as 1.0 μmol/mL tc-LIGRLO-NH_2 was able to elicit a 2.2 fold increase in the release of tryptase. Although the potency of SLIGKV-NH_2 appeared slightly weaker than that of tc-LIGRLO-NH_2, it could still stimulate as much as two fold increase in the release of tryptase from colon mast cells. The reverse peptides tc-OLRGIL-NH_2 and VKGILS –NH_2 had no effect on the release of tryptase when being added at the concentrations from 0.1 to 300 μmol/mL (Figure 1). The quantity of tryptase released elicited by tc-LIGRLO-NH_2 (100 μmol/mL) was similar to that induced by 10 μg/mL anti-IgE or 1 μg/mL calcium ionophore. Trypsin at the concentrations of 1.0-100 μg/mL was capable of provoking a dose dependent release of tryptase with a maximum of 16 ng/mL tryptase being released by 100 μg/mL trypsin (Figure 1). Approximately 80% trypsin induced release of tryptase was diminished by SBTI when trypsin and SBTI were added to cells at the same time (Figure 1).

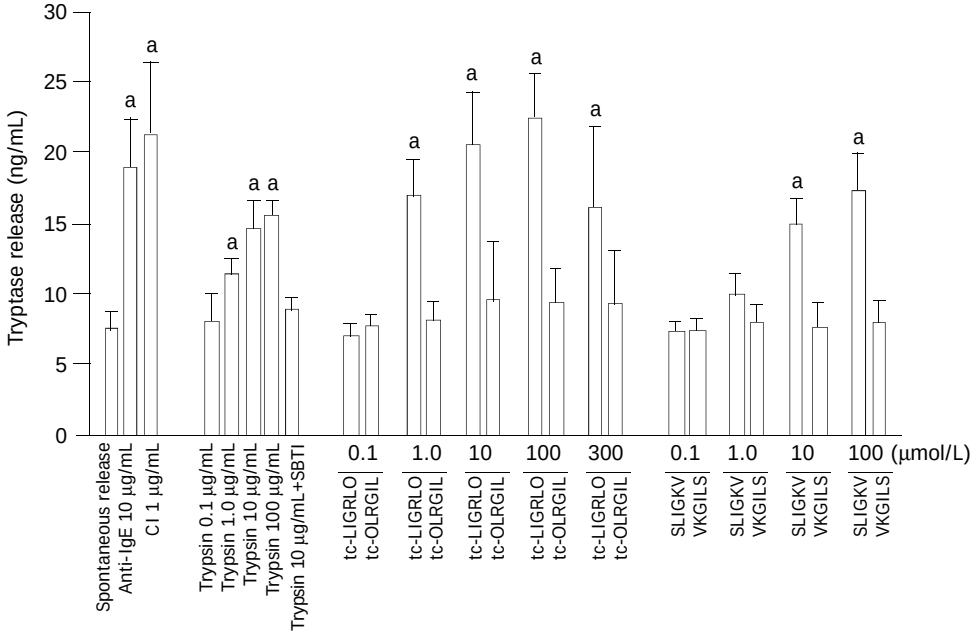

Figure 1 Effects of tc-LIGRLO, tc-OLRGIL, SLIGKV, VKGILS, trypsin, anti-IgE and calcium ionophore A23187 (CI) on tryptase release from colon mast cells. The values shown are mean±SEM for four separate experiments. Stimulus or control was incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with buffer alone group (paired Student's *t* test).

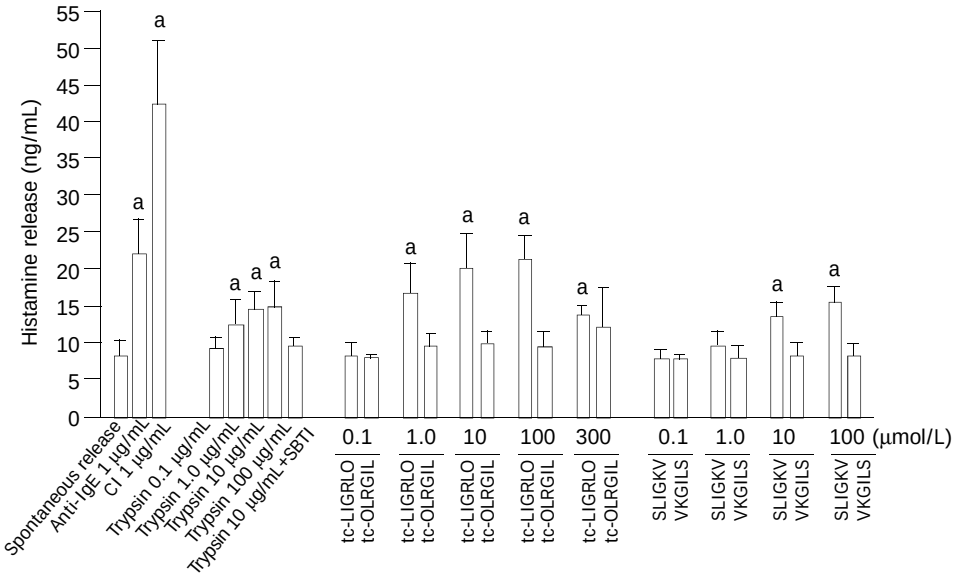

Figure 2 Effects of tc-LIGRLO, tc-OLRGIL, SLIGKV, VKGILS, trypsin, anti-IgE and calcium ionophore A23187 (CI) on histamine release from colon mast cells. The values shown are mean±SEM for four separate experiments. Stimulus or control was incubated with cells for 15 min before termination of the reactions. [a]$P<0.05$ compared with buffer alone group (paired Student's *t* test).

Induction of histamine release by agonists of PAR-2

Both PAR-2 agonists tc-LIGRLO-NH_2 and SLIGKV-NH_2 were also able to induce a dose dependent release of histamine from colon mast cells. More than 2.5 and 2.0 fold increases in histamine release were provoked by 100 μmol/mL tc-LIGRLO-NH_2 and SLIGKV-NH_2, respectively. However, histamine release elicited by tc-LIGRLO-NH_2 (100 μmol/mL) was less than that induced by anti-IgE (10 μg/mL) and only half of that provoked by calcium ionophore (1 μg/mL). The reverse peptides tc-OLRGIL-NH_2 and VKGILS-NH_2 had no effect on the release of histamine when being added at concentrations up to 300 μmol/mL (Figure 2). Trypsin at the concentrations from 1.0 to 100 μg/mL was capable of provoking a dose dependent release of histamine with a maximum of 14 ng/mL histamine being released by 100 μg/mL trypsin. Approximately 70% trypsin induced release of histamine was reduced by SBTI when trypsin and SBTI were added at the same time to cells (Figure 2).

Time course for tc-LIGRLO-NH_2

The release of tryptase and histamine in response to tc-LIGRLO-NH_2 was maximized within 3 min following addition of 100 μmol/mL tc-LIGRLO-NH_2 to colon mast cells. The maximum release of tryptase and histamine was then maintained for at least 15 min (Figure 3). When tc-LIGRLO-NH_2, anti-IgE and calcium ionophore were incubated with cells for a prolonged period of 30 min, the amount of tryptase and histamine released appeared not being increased (Table 1).

Table 1 Effects of tc-LIGRLO, tc-OLRGIL, anti-IgE and calcium ionophore A23187 (CI) on tryptase and histamine release from colon mast cells following 30 min incubation period

Concentration of stimulus		Tryptase (ng/mL)	Histamine (ng/mL)
Buffer alone		8.2±0.6	9.5±1.7
tc-LIGRLO	100 μmol/mL	22±3.0[a]	17±1.3[a]
tc-OLRGIL	100 μmol/mL	8.4±2.8	10±1.9
Anti-IgE	10 μg/mL	19±4.1[a]	19±4.8[a]
CI	1.0 μg/mL	13±0.6[a]	34±4.3[a]

The values shown are mean±SEM for four separate experiments performed in duplicate. [a]$P<0.05$ compared with buffer alone group (paired Student's *t* test).

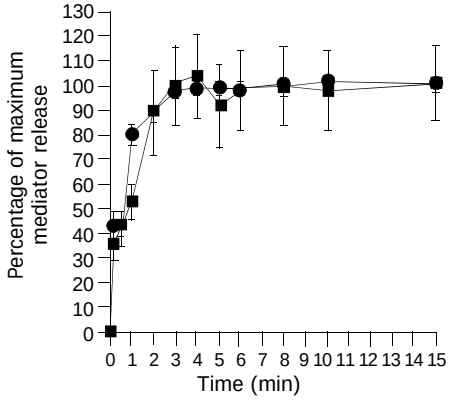

Figure 3 Time course for tc-LIGRLO (100 μM) induced release of tryptase (—■—) and histamine (—●—) from colon mast cells. Data shown are mean±SEM of four separate experiments.

Effects of pertussis toxin and metabolic inhibitors on tryptase and histamine release

Tryptase release induced by tc-LIGRLO-NH_2, SLIGKV-NH_2 and trypsin was abolished by pretreatment of colon mast cells with metabolic inhibitors or pertussis toxin (Table 2). The same treatment was also able to almost completely inhibit histamine release from mast cells (Table 3).

Table 2 Inhibition of tc-LIGRLO, SLIGKV, trypsin, anti-IgE or calcium ionophore A23187 (CI) induced tryptase release from colon mast cells by pertussis toxin (1 μg/mL) and metabolic inhibitors

Concentration of stimulus		% inhibition of tryptase release	
		Pertussis toxin	Metabolic inhibitors
tc-LIGRLO	100 μmol/mL	85±9.6[a]	91±3.9[a]
SLIGKV	100 μmol/mL	91±4.8[a]	94±3.6[a]
Trypsin	10 μg/mL	100±0[a]	95±4.0[a]
Anti-IgE	10 μg/mL	87±10[a]	99±0.5[a]
CI	1.0 μg/mL	84±8.8[a]	89±4.7[a]

The values shown are mean±SEM for four separate experiments performed in duplicate. [a]$P<0.05$ compared with the uninhibited control group (paired Student's *t* test).

Table 3 Inhibition of tc-LIGRLO, SLIGKV, trypsin, anti-IgE or calcium ionophore A23187 (CI) induced histamine release from colon mast cells by pertussis toxin (1 μg/mL) and metabolic inhibitors

Concentration of stimulus		% inhibition of histamine release	
		Pertussis toxin	Metabolic inhibitors
tc-LIGRLO	100 μmol/mL	92±5.4[a]	98±2.0[a]
SLIGKV	100 μmol/mL	95±5.9[a]	92±3.8[a]
Trypsin	10 μg/mL	84±6.8[a]	88±8.7[a]
Anti-IgE	10 μg/mL	84±5.2[a]	99±1.1[a]
CI	1.0 μg/mL	97±2.4[a]	99±0.1[a]

The values shown are mean±SEM for four separate experiments performed in duplicate. [a]$P<0.05$ compared with the uninhibited control group (paired Student's *t* test).

DISCUSSION

The finding in the current study that PAR-2 agonists were able to activate human mast cells fulfilled our hypothesis that human mast cells possess a self-amplification mechanism to IgE dependent activation through their mediator tryptase. That is to say that upon activation, mast cells release tryptase, and the released tryptase then activates their neighbouring mast cells or acts on its host mast cells through PAR-2.

The maximum 22.5 ng/mL tryptase and 21 ng/mL histamine release induced by tc-LIGRLO-NH_2 were comparable to those induced by 10 μg/mL anti-IgE, indicating that the PAR-2 agonist is a potent stimulus of mast cells. SLIGKV was also able to provoke a significant tryptase and histamine release from colon mast cells, but its action appeared slightly weaker than tc-LIGRLO-NH_2. This could be due to the structural difference between the two molecules. As little as 1.0 μmol/mL tc-LIGRLO-NH_2 was able to stimulate a significant release of tryptase and histamine, suggesting further that this compound is a potent mast cell secretagogue, and the concentration of the stimulus is easy to be achieved under physiological conditions. It was little surprised to observe that trypsin was able to activate colon mast cells as there should be a relatively high concentration of trypsin in human intestinal tract, and some of them should be able to penetrate through the epithelial lining of intestine, particularly when paracellular permeability of colon was increased upon PAR-2 activation[4]. Nevertheless, it should be interesting to learn the physiological or pathophysiological role of mast cell activation by trypsin in gastrointestinal tract in future. The fact that reversed peptides of PAR-2 agonists had little effect on tryptase and histamine release from colon mast cells proved further the specificity of the PAR-2 agonists on mast cells. The maximum release of tryptase and histamine from colon mast cells was achieved at 3 min after adding tc-LIGRLO-NH_2 to cells, suggesting that its action on mast cells was slower than that of neuropeptide substance P, which reached peak histamine release within 20 sec of incubation[24], but faster than that induced by anti-IgE and calcium ionophore, which required at least 6 min to complete[21].

The inhibition of trypsin induced tryptase and histamine release by SBTI, a mixed type of inhibitor of tryptic enzymes[25], indicating that the process was not cytotoxic and required an intact catalytic site of trypsin. Pretreatment of cells with metabolic inhibitors antimycin A which blocks the oxidative phosphorylation process of cells, and 2-deoxy-D-glucose which blocks anaerobic metabolism pathway in cells, abolished the actions of tc-LIGRLO-NH_2, SLIGKV, trypsin, anti-IgE as well as calcium ionophore, indicating that the release of tryptase and histamine induced by them was a non-cytotoxic process, and was dependent on cell energy supply. Tryptase and histamine release provoked by tc-LIGRLO-NH_2, SLIGKV, trypsin, anti-IgE and calcium ionophore were also inhibited by pretreatment of cells with pertussis toxin, suggesting that the degranulation process elicited by them was associated with the activation of G-protein coupled receptors[26].

Over the last decade, a number of functions of mast cell tryptase have been discovered. These include induction of microvascular leakage[27], accumulation and activation of eosinophils and neutrophils[28,29], provocation of release of IL-8 from epithelial cells[30]. Similarly, PAR-2 agonists were found to be able to provoke the inflammatory response in the rat paw[31,32] and mediate eosinophil infiltration and hyperreactivity in allergic inflammation of the airway[33]. Of particular importance is the finding that the delayed onset of inflammation was observed in protease-activated receptor-2-deficient mice[34]. These highlighted the crucial role of tryptase and its receptor PAR-2 in inflammation. The finding that PAR-2 agonists were able to activate human colon mast cells in the current study added a new concept to the tryptase and PAR-2 theory, indicating further the importance of them in the pathogenesis of inflammation. In conclusion, the agonists of PAR-2 and trypsin are potent secretagogues of human colon mast cells, which are likely to contribute to the development of inflammatory disorders in human gut.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Inhibition of tryptase release from human colon mast cells by protease inhibitors

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Abstract

AIM: To investigate the ability of protease inhibitors to modulate tryptase release from human colon mast cells.

METHODS: Enzymatically dispersed cells from human colon were challenged with anti-IgE or calcium ionophore A23187 in the absence or presence of tryptase and chymase inhibitors, and tryptase release was determined.

RESULTS: IgE dependent tryptase release from colon mast cells was inhibited by up to approximately 37%, 40% and 36.6% by chymase inhibitors Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPFM), *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK), and α_1-antitrypsin, respectively. Similarly, the inhibitors of tryptase leupeptin, *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and lactoferrin were also able to inhibit anti-IgE induced tryptase release by a maximum of 39.4%, 47.6% and 36.6%, respectively. The inhibitory actions of chymase inhibitors, but not tryptase inhibitors on colon mast cells were enhanced by preincubation of them with cells for 20 min before challenged with anti-IgE. At a concentration of 10 μg/mL, protamine was able to inhibit anti-IgE and calcium ionophore induced tryptase release. However, at 100 μg/mL, protamine elevated tryptase levels in supernatants. A specific inhibitor of aminopeptidase amastatin had no effect on anti-IgE induced tryptase release. The significant inhibition of calcium ionophore induced tryptase release was also observed with the inhibitors of tryptase and chymase examined. The inhibitors tested by themselves did not stimulate tryptase release from colon mast cells.

CONCLUSION: It was demonstrated for the first time that both tryptase and chymase inhibitors could inhibit IgE dependent and calcium ionophore induced tryptase release from dispersed colon mast cells in a concentration dependent of manner, which suggest that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic of colitis in man.

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INTRODUCTION

It has been reported that increased number of mast cells and mast cell degranulation are closely associated with a number of gastrointestinal diseases including idiopathic inflammatory bowel disease[1], chronic ulcerative colitis[2], Crohn' s disease[3-5], gastritis[5] and collagenous colitis[6,7], irritable bowel syndrome[8,9] and chronic inflammatory duodenal bowel disorders[10]. Through releasing their proinflammatory mediators including histamine, tryptase, chymase, heparin and some cytokines[11], mast cells actively participate in the pathogenesis of these intestinal diseases.

Tryptase is a tetrameric serine proteinase that constitutes about 20% of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells[12] in a catalytically active form[13]. Relatively higher secretion of tryptase has been detected in ulcerative colitis[14], implicating that this mediator is involved in the pathogenesis of intestinal diseases. Evidence is emerging that tryptase may be a key mediator of allergic inflammation and a promising target for therapeutic intervention[15] as it has been found to be able to induce microvascular leakage in the skin of guinea pig[16], bronchoconstriction[17] in allergic sheep airways, inflammatory cell accumulation in peritoneum of mouse[18] and release of IL-8 from epithelial cells[19]. Moreover, tryptase has been proved to be a unique marker of mast cell degranulation *in vitro* as it is more selective than histamine to mast cells[20].

In recent years, inhibitors of tryptase[21,22] and chymase[23] were discovered to possess the ability to inhibit histamine release from human skin, tonsil, synovial[24] and colon mast cells[25], suggesting these inhibitors are likely to be developed as a novel class of mast cell stabilizers. However, little is known of the actions of tryptase and chymase inhibitors on tryptase release from human colon mast cells. We therefore investigated the effects of these two groups of inhibitors on IgE dependent or independent tryptase release from human colon mast cells in the current study.

MATERIALS AND METHODS

Dispersion of mast cells

Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removal of fat, tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm^3, and then incubated with 1.5 mg/mL collagenase (Sigma) and 0.75 mg/mL hyaluronidase (Sigma) in minimum essential medium (MEM) containing 2% fetal calf serum (1 g colon/10 mL buffer) for 70 min at 37 ℃. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100 μm in diameter), washed and maintained in MEM (Gibco) (containing 10% FCS, 200 U/mL penicillin, 200 μg/mL streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after stained by alcine blue, ranged from 3.5% to 5.4%.

Mast cell challenge

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with $CaCl_2$ and $MgCl_2$ (complete HBSS), and 100 μL aliquots containing 4-6×10^3 mast cells

were added to a 50 μL anti-IgE (Serotec, UK), calcium ionophore (Sigma), or inhibitor in complete HBSS and incubated for 15 min at 37 ℃. The reaction was terminated by addition of 150 μL ice cold incomplete HBSS and the tubes were centrifuged immediately (500 *g*, 10 min, 4 ℃). All experiments were performed in duplicate. Supernatants were stored at -20 ℃ until tryptase concentrations were determined.

Inhibition of release of tryptase

For some experiments, protease inhibitor was preincubated with cells for 20 min before anti-IgE or calcium ionophore was added. Protease inhibitor and anti-IgE or calcium ionophore were also added to cells at the same time (no preincubation period). Data were expressed as the percentage inhibition of tryptase release, taking into account tryptase release in the presence and absence of the inhibitor. As for our previous experiments, the optimal tryptase release from colon mast cells was induced by 10 μg/mL anti-IgE or 1 μg/mL calcium ionophore[20], and therefore they were chosen as standard concentrations throughout the study.

Tryptase measurement

Tryptase concentrations were measured with a sandwich ELISA procedure with a specific polyclonal antibody against human tryptase as the capture antibody and AA5 a monoclonal antibody specific for human tryptase as the detecting antibody[26].

Statistical analyses

Statistical analyses were performed with SPSS software. Data were expressed as mean±SEM. Analysis of variance indicated significant differences between groups with ANOVA. For the preplanned comparison of interest, Student' s *t* test was applied. For all analyses, $P<0.05$ was taken as statistically significant.

RESULTS

Effects of secretagogues and inhibitors on tryptase release from mast cells

At 15 min following incubation, anti-IgE at 10 μg/mL and calcium ionophore at 1 μg/mL were able to induce 41.6±4.3 ng/mL and 38.8±3.0 ng/mL tryptase release from colon mast cells, respectively, whereas at the same time point spontaneous tryptase release (buffer alone) was 22.4±3.2 ng/mL. The same concentrations of anti-IgE and calcium ionophore were also able to provoke a significant tryptase release from colon mast cells following a 35 min incubation period (Table 1). All protease inhibitors tested had no stimulatory effect on colon mast cells following a 15 min or a 35 min incubation period (data not shown).

Table 1 Spontaneous and anti-IgE or calcium ionophore induced tryptase release from human colon mast cells

Compound		Tryptase released (ng/mL)	
		15 min	35 min
Buffer alone		22.4±3.2	20.5±2.8
Anti-IgE	10 μg/mL	41.6±4.3[a]	37.6±2.6[a]
CI	1.0 μg/mL	38.8±3.0[a]	38.4±3.6[a]

The values shown are mean±SEM for six separate experiments. [a]$P<0.05$ compared with buffer alone control (Student's *t* test).

Inhibition of anti-IgE induced tryptase release from mast cells

The concentration dependent inhibition of anti-IgE induced release of tryptase from colon mast cells was observed when anti-IgE and various concentrations of chymase inhibitors ZIGPFM, TPCK, and α_1-antitrypsin were added to cells at the same time. Up to approximately 37%, 40% and 36.6% inhibition of IgE dependent tryptase release were achieved with ZIGPFM, TPCK, and α_1-antitrypsin, respectively (Figure 1). As little as 10 ng/mL ZIGPFM was able to significantly inhibit IgE dependent tryptase release. Preincubation of ZIGPFM and TPCK with cells for 20 min before challenged with anti-IgE was able to moderately enhance their inhibitory actions on cells (Figure 2).

The inhibitors of tryptase leupeptin, TLCK and lactoferrin were also able to inhibit anti-IgE induced tryptase release in a concentration dependent manner, and a maximum of some 39.4%, 47.6%, and 36.6% of inhibition was achieved with 200 μmol/mL leupeptin, 100 μmol/mL TLCK, and 30 μmol/mL lactoferrin, respectively (Figure 1). Preincubation of inhibitors of tryptase with cells for 20 min before anti-IgE was added had little effect on their abilities to inhibit anti-IgE induced tryptase release (Figure 2). A specific inhibitor of aminopeptidase, amastatin had no effect on anti-IgE induced tryptase release. When 10 μg/mL protamine was added to cells at the same time with anti-IgE, or when 1.0 μg/mL protamine was preincubated with cells before addition of the stimulus, anti-IgE induced tryptase release was significantly inhibited. However, when 100 μg/mL protamine and anti-IgE was added to cells at the same time, tryptase concentrations measured in cell supernatants were much higher than those induced by anti-IgE alone (Table 2).

Table 2 Effect of protamine on anti-IgE or calcium ionophore (CI) induced tryptase release from human colon mast cells

Protamine concentration (μg/mL)	% inhibition of tryptase release			
	No preincubation		20 min preincubation	
	Anti-IgE	CI	Anti-IgE	CI
0.1	4.0±2.3	5.1±4.5	nd	nd
1.0	17.3±5.2	16.7±4.6	21.4±3.8[a]	19.5±2.9[a]
10	20.9±4.3[a]	17.1±3.8	-0.7±1.8	-9.7±11
100	-54.7±13[b]	-24.9±8.9[b]	nd	nd

The values shown are mean±SEM for six separate experiments. Protamine was either added to cells at the same time with anti-IgE or CI, or preincubated with cells for 20 min before challenged with anti-IgE or CI. [a]levels of tryptase reduced in comparison with the uninhibited control ($P<0.05$, Student's *t* test). [b]levels of tryptase elevated in comparison with the uninhibited control ($P<0.05$, Student's *t* test). nd=not done.

Inhibition of calcium ionophore induced tryptase release from mast cells

The concentration dependent inhibition of calcium ionophore induced tryptase release from colon mast cells was observed when calcium ionophore and various concentrations of chymase inhibitors ZIGPFM, TPCK, and α_1-antitrypsin were added to cells at the same time. Up to approximately 27.6%, 35.3% and 23% inhibition of IgE dependent tryptase release were achieved with ZIGPFM, TPCK, and α_1-antitrypsin, respectively (Figure 3). Preincubation of the inhibitors with cells for 20 min before challenged with calcium ionophore enhanced the inhibitory ability of ZIGPFM, but not TPCK (Figure 4).

Calcium ionophore stimulated tryptase release was also reduced by addition of the various concentrations of inhibitors of tryptase to cells. Leupeptin, TLCK and lactoferrin were able to inhibit calcium ionophore stimulated tryptase release by up to approximately 27.1%, 44.1% and 38.2% respectively, when they were added to cells together with calcium ionophore (Figure 3). The extent of inhibition by leupeptin and TLCK was not affected by preincubation of them with cells for 20 min before calcium ionophore was added (Figure 4). Protamine at a concentration of 1 μg/mL was also able to inhibit calcium ionophore induced tryptase release (Table 2).

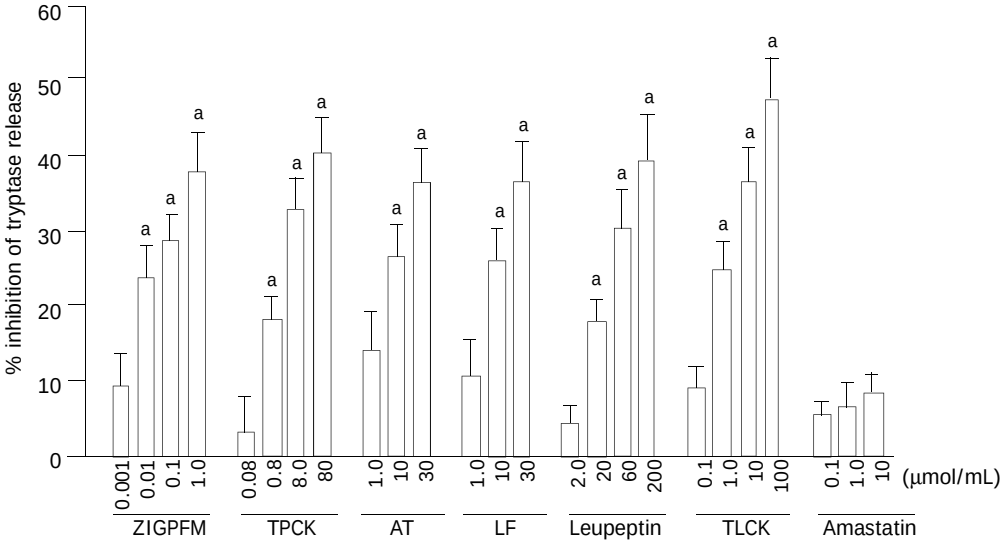

Figure 1 Inhibition of anti-IgE (10 μg / mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. $^aP<0.05$ compared with the responses with uninhibited controls. AT=α_1-antitrypsin; LF=lactoferrin.

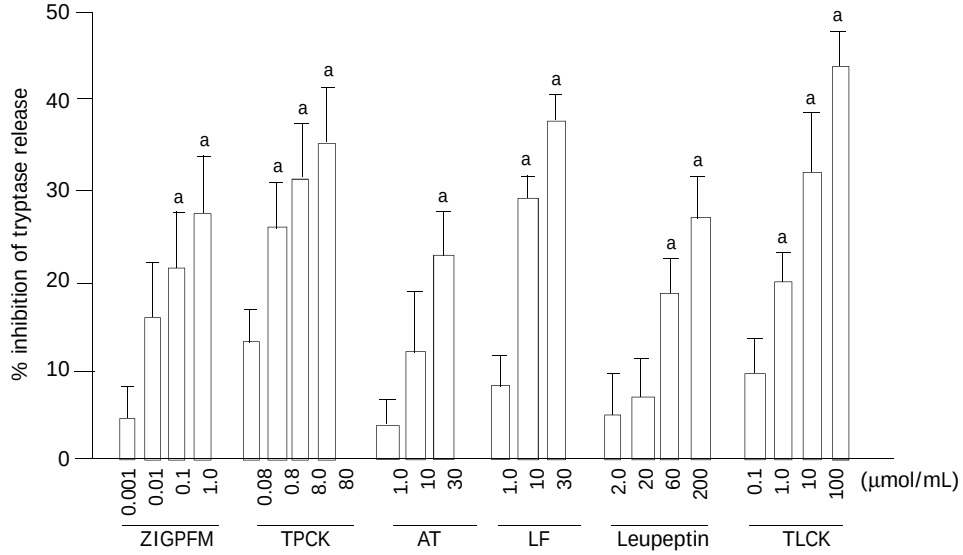

Figure 2 Inhibition of anti-IgE (10 μg / mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors were preincubated with cells for 20 min before anti-IgE was added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. $^aP<0.05$ compared with the responses of uninhibited controls.

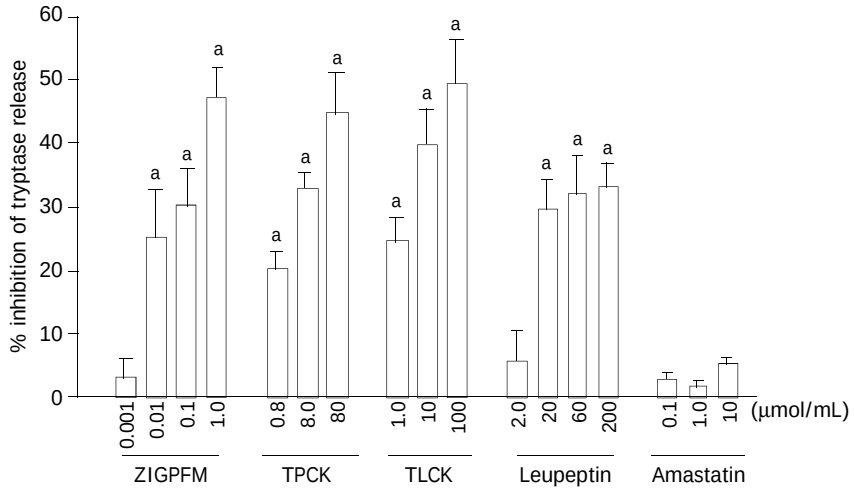

Figure 3 Inhibition of calcium ionophore (1 μg / mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. $^aP<0.05$ compared with the responses with uninhibited controls. AT=α_1-antitrypsin; LF=lactoferrin.

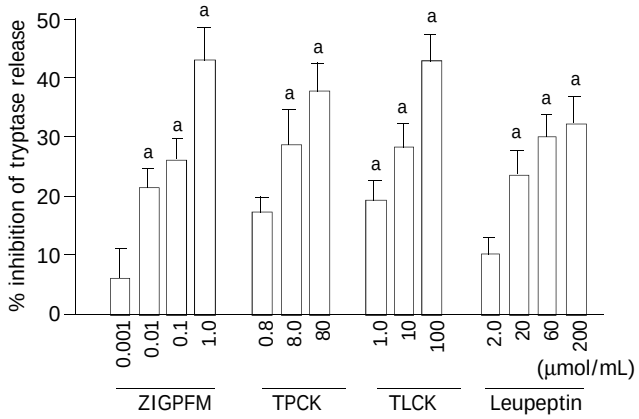

Figure 4 Inhibition of calcium ionophore (1 μg / mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors were preincubated with cells for 20 min before calcium ionophore was added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. [a]$P<0.05$ compared with the responses of uninhibited controls.

DISCUSSION

We have found for the first time that inhibitors of tryptase and chymase were able to inhibit anti-IgE and calcium ionophore induced tryptase release from dispersed human colon mast cells, which may indicate a potential of a novel therapy for inflammatory bowel disease or other mast cell related intestinal diseases.

Up to approximately 40% inhibition of IgE dependent tryptase release from colon mast cells was observed with inhibitors of chymase, indicating that a chymase activity was involved in the process of IgE dependent gut mast cell degranulation. This was consistent with our previous finding that chymase inhibitors inhibited IgE dependent histamine release, which has indirectly proved that tryptase and histamine are likely to share a similar degranulation process. Unlike inhibition of histamine release, preincubation of ZIGPFM and TPCK with cells for 20 min before challenged with anti-IgE appeared to further reduce the quantity of tryptase released from mast cells, implicating that there may be some difference between tryptase and histamine release processes. Similar to chymase inhibitors, tryptase inhibitors inhibited up to some 47.6% anti-IgE induced tryptase release from colon mast cells, which implicated that a tryptase activity was likely to be involved in the process of colon mast cell degranulation. Once again, this was consistent with our previous finding that tryptase inhibitors were able to inhibit IgE dependent histamine release.

Since the majority of these inhibitors at the concentrations used in the current study were able to inhibit more than 95% tryptase or chymase activity in enzyme assays[27], the incomplete inhibition of tryptase release from mast cells may suggest that some pathways other than tryptase and chymase pathways are involved in the anti-IgE induced degranulation of gut mast cells. A specific inhibitor of aminopeptidase amastatin, which did not inhibit chymotrypsin and trypsin activities[28], was used as an irrelevant protease inhibitor control. It had no significant effects on anti-IgE induced tryptase release from colon mast cells, which proved the specificity of actions of tryptase and chymase inhibitors on tryptase release from mast cells.

Calcium ionophore is a calcium carrier that could help to elevate the calcium concentration in cytoplasms of mast cells[29], and therefore acts on the downstream site of the process of mast cell degranulation. The inhibition of calcium ionophore induced tryptase release by the inhibitors of tryptase and chymase in the current study might also suggest the involvement of tryptase and chymase activities in mast cell degranulation process was at the downstream site and most likely after influx of calcium ions into mast cells. The evidence that tryptase and chymase are sited in the granules of mast cells in their fully active form supports further the likelihood that these two mast cell serine proteases are involved in IgE dependent activation of colon mast cells. The results that tryptase levels were elevated when 100 μg/mL protamine was added to cells at the same time with unexpected anti-IgE. This was most likely due to the tetrameric structure of tryptase being dissociated by protamine[30], thus more tryptase monomers existed in supernatants, and were recognized by AA5 as an intact tryptase molecule.

Some of the latest reports on tryptase inhibitors demonstrated the importance of these potential anti-inflammatory drugs. Inhaled APC366 was able to attenuate allergen-induced late-phase airway obstruction in asthma[31], and APC2059 could improve the symptomatic scores of patients with mildly to moderately active ulcerative colitis in an open-label pilot study[32]. Our findings in the current study may at least partially explain why tryptase inhibitors could treat these diseases. Moreover, the successful treatment of acute ulcerative colitis[33] and Crohn's disease[34] with mast cell stabilizer ketotifen further strongly suggests that inhibitors of tryptase and chymase are likely to become a novel class of anti-inflammatory drugs with their anti-inflammatory actions and mast cell stabilizing properties.

In conclusion, the inhibitors of both tryptase and chymase are able to inhibit anti-IgE dependent and calcium ionophore induced tryptase release from colon mast cells, indicating that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic colitis in man.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Modulation of histamine release from human colon mast cells by protease inhibitors

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Abstract

AIM: To investigate the ability of protease inhibitors to modulate histamine release from human colon mast cells.

METHODS: Enzymatically dispersed cells from human colon were challenged with anti-IgE or calcium ionophore A23187 in the absence or presence of tryptase and chymase inhibitors, and histamine release was determined.

RESULTS: IgE dependent histamine release from colon mast cells was inhibited by up to approximately 37%, 26% and 36.8% by chymase inhibitors Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPFM), N-Tosyl-L-phenylalanyl-chloromethyl ketone (TPCK), and α_1-antitrypsin, respectively. Similarly, inhibitors of tryptase leupeptin, N-tosyl-L-lysine chloromethyl ketone (TLCK), lactoferrin and protamine were also able to inhibit anti-IgE induced histamine release by a maximum of some 48%, 37%, 40% and 34%, respectively. Preincubation of these inhibitors with cells for 20 min before challenged with anti-IgE had small effect on the inhibitory actions of these inhibitors on colon mast cells. A specific inhibitor of aminopeptidase amastatin had no effect on anti-IgE induced histamine release. The significant inhibition of calcium ionophore induced histamine release was also observed with the inhibitors of tryptase and chymase examined. Apart from leupeptin and protamine, the inhibitors tested by themselves did not stimulate colon mast cells.

CONCLUSION: It was demonstrated that both tryptase and chymase inhibitors could inhibit IgE dependent and calcium ionophore induced histamine release from dispersed colon mast cells in a concentration dependent of manner, which suggest that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic of colitis in man.

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INTRODUCTION

It has been reported that mast cells and their inflammatory mediators are closely associated with a number of intestinal diseases including idiopathic inflammatory bowel disease[1], chronic ulcerative colitis[2], Crohn's disease[3] and collagenous colitis[4]. Through release their proinflammatory mediators including histamine, tryptase, chymase, heparin and some cytokines[5], mast cells actively participate in the pathogenesis of these intestinal diseases.

As a proinflammatory mediator, histamine is selectively located in the granules of human mast cells and basophils and released from these cells upon degranulation. To date, a total of four histamine receptors H_1, H_2, H_3 and H_4 have been discovered[6] and the first three of them are located in human gut[7,8], which prove that there are some specific targets that histamine can work on in intestinal tract. Indeed, increased levels of histamine or enhanced histamine metabolism have been observed in collagenous colitis, food allergy[9], Crohn's disease[10], ulcerative colitis[10,11] and allergic enteropathy[11], indicating that this mediator is involved in the pathogenesis of these diseases.

For more than four decades, histamine has been widely used as a marker of mast cell degranulation *in vitro*, and numerous anti-allergic drugs such as sodium cromoglycate, lodoxamide, salbutamal, ketotifen, terfenadine and cetirizine[12,13] and salmeteral[14] were reported to be able to inhibit anti-IgE induced histamine release from human mast cells. In recent years, inhibitors of tryptase[15,16] and chymase[17] have been discovered to possess the ability to inhibit histamine release from human skin, tonsil and synovial mast cells[18], suggesting these inhibitors are likely to be developed as a novel class of mast cell stabilizers. However, little is known of the actions of tryptase and chymase inhibitors on histamine release from human colon mast cells. We therefore investigated the effects of these two groups of inhibitors on IgE dependent or independent histamine release from human colon mast cells in the current study.

MATERIALS AND METHODS

Dispersion of mast cells

Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removal of fat, tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm^3, and then incubated with 1.5 mg/mL collagenase (Sigma) and 0.75 mg/mL hyaluronidase (Sigma) in minimum essential medium (MEM) containing 2% fetal calf serum (1 g colon/10 mL buffer) for 70 min at 37 ℃. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100 μm diameter), washed and maintained in MEM (Gibco) (containing 10% FCS, 200 U/mL penicillin, 200 μg/mL streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after stained by alcine blue, ranged from 3.5% to 5.4%.

Mast cell challenge

The challenge procedure was performed as described previously[19]. Dispersed cells were resuspended in HEPES

buffered salt solution (HBSS, pH 7.4) with $CaCl_2$ and $MgCl_2$ (complete HBSS), and 100 μL aliquots containing 4-6×10^3 mast cells were added to a 50 μL anti-IgE (Serotec, UK), calcium ionophore (Sigma), or inhibitor in complete HBSS and incubated for 15 min at 37 ℃. The reaction was terminated by addition of 150 μL ice cold incomplete HBSS and the tubes were centrifuged immediately (500 *g*, 10 min, 4 ℃). All experiments were performed in duplicate. For the measurement of total histamine concentration, in certain tubes the suspension was boiled for 6 min. Supernatants were stored at -20 ℃ until histamine concentrations were determined.

Inhibition of release of histamine

For some experiments, protease inhibitor was preincubated with cells for 20 min before anti-IgE or calcium ionophore being added. Protease inhibitor and anti-IgE or calcium ionophore were also added to cells at the same time (no preincubation period). Data were expressed as the percentage of inhibition of histamine release, taking into account of histamine release in the presence and absence of the inhibitor. As for our previous experiments, the optimal histamine release from colon mast cells was induced by 10 μg/mL anti-IgE or 1 μg/mL calcium ionophore[20], and therefore they were chosen as standard concentrations throughout the study.

Histamine measurement

Histamine concentrations were determined using a glass fibre-based fluorometric assay[15]. The procedure involved the binding of histamine to a glass-fiber matrix (RafLab, Copenhagen, Denmark) and its detection spectrophotometrically with Perkin-Elmer LS 2 detector (Denmark) following addition of o-phthaldialdehyde (OPT). Histamine release was expressed as a percentage of total cellular histamine levels, and corrected for the spontaneous release measured in tubes in which cells had been incubated with the HBSS diluent alone.

Statistical analyses

Statistical analyses were performed with SPSS software. Data were expressed as mean±SEM. Where analysis of variance indicated significant differences between groups with ANOVA, for the preplanned comparisons of interest, Student's *t* test was applied. For all analyses, $P<0.05$ was taken as statistically significant.

RESULTS

Effects of protease inhibitors on histamine release from mast cells

At 15 min following incubation, leupeptin at concentration 200 μmol/mL and protamine at 100 μg/mL were able to provoke small but nevertheless significant histamine release from colon mast cells (Table 1). The same concentration of leupeptin was also capable of eliciting histamine release following a 35 min incubation period (Table 2). All the other protease inhibitors tested had no stimulatory action on colon mast cells. Leupeptin and protamine at all other concentrations did not induce a significant histamine release from colon mast cells. In the same experiments, anti-IgE and calcium ionophore were able to induce up to 11% and 21.8% net histamine release, respectively.

Inhibition of anti-IgE induced histamine release from mast cells

The concentration dependent inhibition of anti-IgE induced release of histamine from colon mast cells was observed when anti-IgE and various concentrations of chymase inhibitors ZIGPFM, TPCK, and α_1-antitrypsin were added to cells at the same time. Up to approximately 37%, 26% and 36.8% inhibition of IgE dependent histamine release were achieved with ZIGPFM, TPCK, and α_1-antitrypsin, respectively (Figure 1). Preincubation of ZIGPFM and TPCK with cells for 20 min before challenged with anti-IgE was able to slightly enhance their inhibitory actions (Figure 2).

The inhibitors of tryptase leupeptin, TLCK, lactoferrin and protamine were also able to inhibit anti-IgE induced histamine release in a concentration dependent manner, and a maximum of 48%, 37%, 40% and 34% inhibition was achieved with 200 μmol/mL leupeptin, 100 μmol/mL TLCK, 30 μmol/mL lactoferrin and 100 μg/mL protamine, respectively (Figure 1). In contrast to inhibitors of chymase, preincubation of inhibitors of tryptase with cells for 20 min before the addition of anti-IgE had little effect on their abilities to inhibit anti-IgE induced histamine release (Figure 2). A specific inhibitor of aminopeptidase, amastatin had no effect on anti-IgE induced histamine release (data not shown).

Table 1 The effects of protease inhibitors on histamine release from human colon mast cells at 15 min incubation period

Compound	Concentration	Net histamine release (%)
ZIGPFM	0.001 μmol / mL	-0.9±0.8
	0.01 μmol / mL	0±1.1
	0.1 μmol / mL	0.8±0.6
	1.0 μmol / mL	0.2±1.2
TPCK	0.08 μmol / mL	3.1±2.0
	0.8 μmol / mL	2.1±1.0
	8.0 μmol / mL	2.0±1.2
	80 μmol / mL	0.7±1.1
α1-antitrypsin	1.0 μmol / mL	0.9±0.5
	10 μmol / mL	1.1±0.6
	30 μmol / mL	1.5±0.6
Lactoferrin	1.0 μmol / mL	-1.0±0.8
	10 μmol / mL	0.8±0.6
	30 μmol / mL	0.5±1.0
TLCK	0.1 μmol / mL	1.7±2.1
	1.0 μmol / mL	1.1±0.6
	10 μmol / mL	2.7±1.7
	100 μmol / mL	3.5±1.2
Amastatin	0.1 μmol / mL	1.5±0.4
	1.0 μmol / mL	2.3±0.6
	10 μmol / mL	3.9±0.6
Leupeptin	2.0 μmol / mL	1.2±0.7
	20 μmol / mL	2.0±1.4
	60 μmol / mL	0.6±0.5
	200 μmol / mL	4.7±0.8[a]
Protamine	0.1 μg / mL	1.7±1.3
	1.0 μg / mL	1.9±1.4
	10 μg / mL	0.2±2.1
	100 μg / mL	4.5±0.5[a]
Anti-IgE	10 μg / mL	11±2.7[a]
Calcium ionophone	1.0 μg / mL	18±3.6[a]

The values shown are mean±SEM for four to six separate experiments. Cells were incubated with each concentration of the compound for 15 min at 37 ℃. Spontaneous histamine release from these cells was 9.2±1.3%. [a]$P<0.05$ compared with buffer alone control (Student's *t* test).

Table 2 The effects of protease inhibitors on histamine release from human colon mast cells at 35 min incubation period

Compound	Concentration	Net histamine release (%)
ZIGPFM	0.001 μmol / mL	0.3±0.3
	0.01 μmol / mL	1.0±0.8
	0.1 μmol / mL	1.8±1.3
	1.0 μmol / mL	1.9±0.6
TPCK	0.8 μmol / mL	0.9±2.1
	8.0 μmol / mL	1.8±1.3
	80 μmol / mL	1.7±3.6
TLCK	1.0 μmol / mL	0±2.4
	10 μmol / mL	0.7±1.9
	100 μmol / mL	0.7±2.2
Amastatin	0.1 μmol / mL	1.0±1.7
	1.0 μmol / mL	-1.9±3.3
	10 μmol / mL	0±3.6
Leupeptin	2.0 μmol / mL	1.0±0.7
	20 μmol / mL	1.2±0.7
	60 μmol / mL	0±0.7
	200 μmol / mL	5.1±0.5[a]
Protamine	1.0 μg / mL	1.2±2.9
	10 μg / mL	1.0±2.5
Anti-IgE	10 μg / mL	10.1±2.5[a]
Calcium ionophone	1.0 μg / mL	21.8±3.8[a]

The values shown are mean±SEM for four to six separate experiments. Spontaneous histamine release from these cells was 9.8±1.7%. [a]$P<0.05$ compared with buffer alone control (Student's *t* test).

Inhibition of calcium ionophore induced histamine release from mast cells

The concentration dependent inhibition of calcium ionophore induced histamine release from colon mast cells was observed when calcium ionophore and various concentrations of chymase inhibitors ZIGPFM, TPCK, and α_1-antitrypsin were added to cells at the same time. Up to approximately 35%, 24% and 23.6% inhibition of IgE dependent histamine release were achieved with ZIGPFM, TPCK, and α_1-antitrypsin, respectively (Figure 3). Preincubation of TPCK with cells for 20 min before challenged with calcium ionophore slightly enhanced its inhibitory ability, whereas the same treatment did not improve the inhibitory ability of ZIGPFM (Figure 4).

Calcium ionophore stimulated histamine release was also reduced by addition of the various concentrations of inhibitors of tryptase to cells. Leupeptin, TLCK, lactoferrin and protamine were able to inhibit calcium ionophore stimulated histamine release by up to approximately 25%, 26%, 25% and 32%, respectively when they were added to cells together with calcium ionophore (Figure 3). The extent of inhibition by leupeptin and TLCK was increased when colon mast cells were preincubated with them for 20 min before calcium ionophore was added. However, the same treatment failed to improve the inhibitory action of protamine (Figure 4).

DISCUSSION

We have found that inhibitors of tryptase and chymase were able to inhibit anti-IgE and calcium ionophore induced histamine release from dispersed human colon mast cells, which may indicate a potential of a novel therapy for inflammatory bowel disease or other mast cell related intestinal diseases.

Up to approximately 37% inhibition of IgE dependent histamine release from colon mast cells was observed with inhibitors of chymase, indicating that a chymase activity was involved in the process of IgE dependent gut mast cell degranulation. This was consistent with our previous findings with human skin, lung[17] and synovium tissues[18], which demonstrated that chymase was involved in the mast cell activation-degranulation process. Comparing mast cells from different human tissues, the order of extent of maximum inhibition by chymase inhibitors was skin (82%) > lung (80%) > synovium (69%) >colon (37%). This might represent a novel type of mast cell heterogeneity, and could also be resulted from an inhibitor of chymase, chymostatin was not used for colon cells, but for the cells from other tissues.

Similar to chymase inhibitors, tryptase inhibitors inhibited

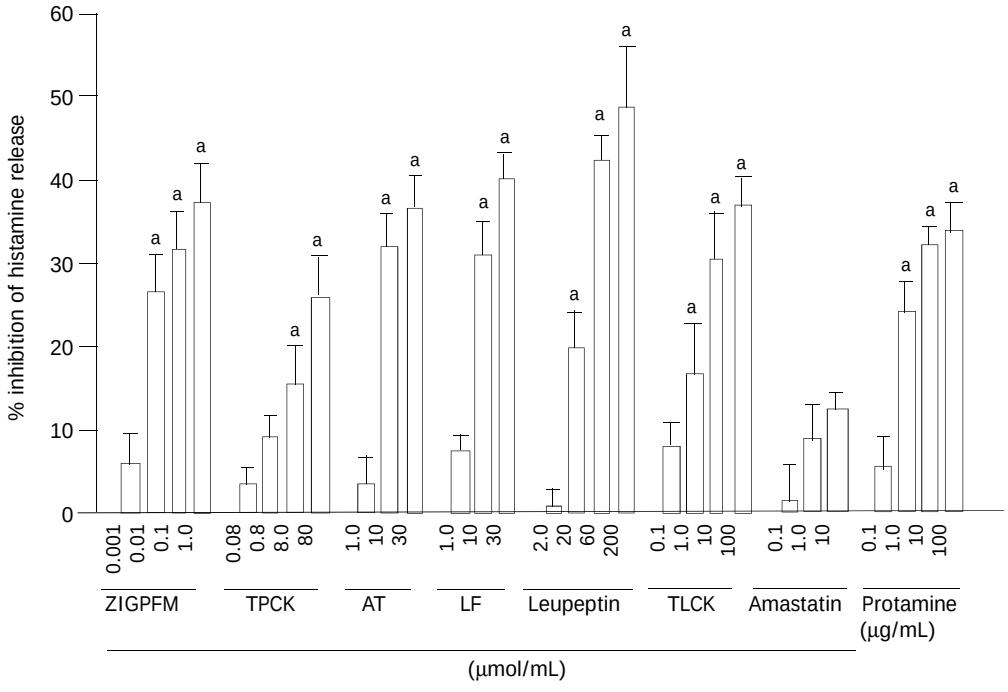

Figure 1 Inhibition of anti-IgE (10 μg / mL) induced histamine release from dispersed colon mast cells by the protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. [a]$P<0.05$ compared with the responses with uninhibited controls. AT=α_1-antitrypsin; LF=lactoferrin.

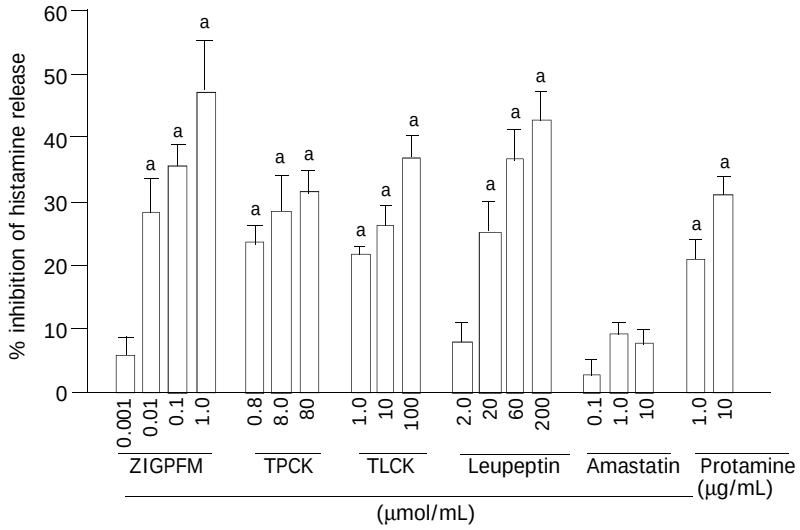

Figure 2 Inhibition of anti-IgE (10 μg / mL) induced histamine release from dispersed colon mast cells by the protease inhibitors. The inhibitors were preincubated with cells for 20 min before anti-IgE was added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. [a]$P<0.05$ compared with the responses with uninhibited controls.

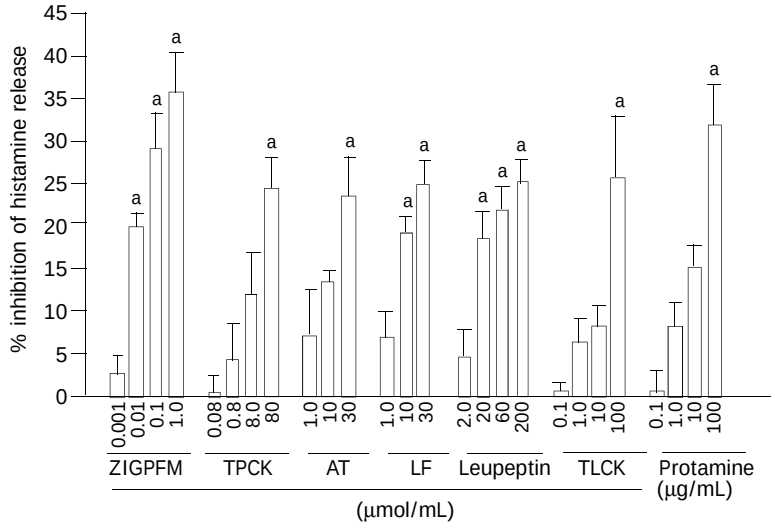

Figure 3 Inhibition of calcium ionophore (1 μg / mL) induced histamine release from dispersed colon mast cells by the protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. [a]$P<0.05$ compared with the responses with uninhibited controls. AT=α_1-antitrypsin; LF=lactoferrin.

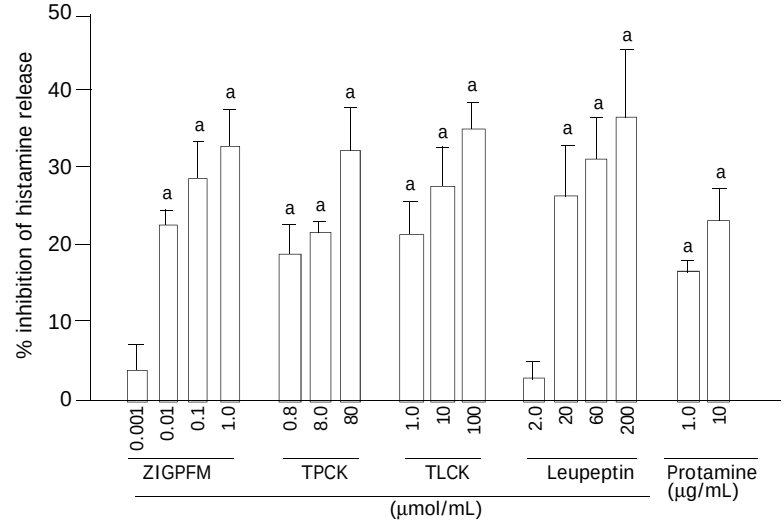

Figure 4 Inhibition of calcium ionophore (1 μg / mL) induced histamine release from dispersed colon mast cells by the protease inhibitors. The inhibitors were preincubated with cells for 20 min before calcium ionophore being added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. [a]$P<0.05$ compared with the responses with uninhibited controls.

up to some 48% anti-IgE induced histamine release from colon mast cells, which implicated that a tryptase activity was likely to be involved in the process of colon mast cell degranulation. Once again, this was consistent with our previous findings with cells from other human tissues including skin, tonsil[15] and synovium[18]. Comparing mast cells from different human tissues, the order of extent of maximum inhibition by tryptase inhibitors was skin (90%) > synovium (70%) > colon (48%) > tonsil (35%).

Since the majority of these inhibitors at the concentrations used in the current study were able to inhibit more than 95% tryptase or chymase activity in enzyme assays[20], the incomplete inhibition of histamine release from mast cells may suggest that some pathways other than tryptase and chymase pathways are involved in the anti-IgE induced degranulation of gut mast cells. A specific inhibitor of aminopeptidase amastatin, which did not inhibit chymotrypsin and trypsin activities[21], was used as an irrelevant protease inhibitor control. It had no significant effects on anti-IgE induced histamine release from colon mast cells, which proved the specificity of actions of tryptase and chymase inhibitors on mast cells. The observation that preincubation of the inhibitors with cells for 20 min before challenged with anti-IgE had little impact on inhibition of IgE dependent histamine release was unexpected, nevertheless it may suggest that the actions of these inhibitors are rather rapid and the involvement of tryptase and chymase activities in anti-IgE induced histamine release is likely at the downstream site of the degranulation process. Since calcium ionophore is a calcium carrier that can help to elevate the calcium concentration in cytoplasms of mast cells, and therefore acts on the downstream site of the process of mast cell degranulation, the inhibition of calcium ionophore induced histamine release by the inhibitors of tryptase and chymase in the current study might also suggest that the involvement of tryptase and chymase activities in mast cell degranulation process was at the downstream site and most likely after influx of calcium ions into mast cells. The evidence that tryptase and chymase are sited in the granules of mast cells in their fully active form supports further the likelihood that these two mast cell serine proteases are involved in IgE dependent activation of colon mast cells.

Over the years, many compounds including sodium cromoglycate, lodoxamide, salbutamal, ketotifen, terfenadine and cetirizine have been recognized as mast cell stabilizers or histamine receptor antagonists, and used as anti-allergic drugs in clinical practice. However, only less than 40% inhibition of IgE dependent mast cell degranulation could be achieved with these compounds, much less than that with inhibitors of tryptase and chymase in the similar experimental system[15,17]. Moreover, mast cell stabilizer drug ketotifen was successfully used to treat acute ulcerative colitis[22] and Crohn' s disease[23]. These strongly suggest that inhibitors of tryptase and chymase are likely to become a novel class of anti-inflammatory drugs with their anti-inflammatory actions and mast cell stabilizing properties.

In conclusion, inhibitors of both tryptase and chymase are able to inhibit anti-IgE dependent and calcium ionophore induced histamine release from colon mast cells, indicating that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic colitis in man.

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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Cloning and expression of human colon mast cell carboxypeptidase

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Abstract

AIM: To clone and express the human colon mast cell carboxypeptidase (MC-CP) gene.

METHODS: Total RNA was extracted from colon tissue, and the cDNA encoding human colon mast cell carboxypeptidase was amplified by reverse-transcription PCR (RT-PCR). The product cDNA was subcloned into the prokaryotic expression vector pMAL-c2x and eukaryotic expression vector pPIC9K to construct prokaryotic expression vector pMAL/human MC-CP (hMC-CP) and eukaryotic pPIC9K/hMC-CP. The recombinant fusion protein expressed in *E.coli* was induced with IPTG and purified by amylose affinity chromatography. After digestion with factor Xa, recombinant hMC-CP was purified by heparin agarose chromatography. The recombinant hMC-CP expressed in *Pichia pastoris* (*P.pastoris*) was induced with methanol and analyzed by SDS-PAGE, Western blot, N-terminal amino acid sequencing and enzyme assay.

RESULTS: The cDNA encoding the human colon mast cell carboxypeptidase was cloned, which had five nucleotide variations compared with skin MC-CP cDNA. The recombinant hMC-CP protein expressed in *E.coli* was purified with amylose affinity chromatography and heparin agarose chromatography. SDS-PAGE and Western blot analysis showed that the recombinant protein expressed by *E. coli* had a molecular weight of 36 kDa and reacted to the anti-native hMC-CP monoclonal antibody (CA5). The N-terminal amino acid sequence confirmed further the product was hMC-CP. *E. coli* generated hMC-CP showed a very low level of enzymatic activity, but *P. pastoris* produced hMC-CP had a relatively high enzymatic activity towards a synthetic substrate hippuryl-L-phenylalanine.

CONCLUSION: The cDNA encoding human colon mast cell carboxypeptidase can be successfully cloned and expressed in *E.coli* and *P. pastoris*, which will contribute greatly to the functional study on hMC-CP.

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INTRODUCTION

Mast cells and their inflammatory mediators have been implicated to play a pivotal role in intestinal diseases such as inflammatory bowel disease[1-7], collagenous colitis[8,9], intestinal anaphylaxis[10,11] and irritable bowel syndrome[11,12]. Mast cell neutral proteases constitute more than 50% granule proteins in mast cells. They are tryptase, MC-CP, chymase, and a cathepsin G-like protease[13-16]. Upon degranulation, these neutral proteases are released and carry out numerous functions in tissues nearby or distant as pro-inflammatory mediators. Recently it was found that mast cell products tryptase and histamine might play an important role in the amplification of degranulation signals in human[17-21].

hMC-CP is a distinctive carboxypeptidase, which is exclusively located in MC_{TC} mast cells, possesses pancreatic carboxypeptidase A (CPA)-like activity, but has a closer amino acid sequence identical to carboxypeptidase B (CPB)[13,22]. The evolution analysis demonstrated that MC-CP originated from a gene duplication along the pancreatic CPB lineage rather than along the pancreatic CPA lineage[22,23]. Although hMC-CP has been detected in skin, lung, and intestinal tissues with immunohistochemistry[13,22,24], and hMC-CP genes from skin and lung were cloned[22,23,25], hMC-CP gene from intestinal tissue is still unknown.

Investigations on the structures and functions of human tryptase and chymase have made impressive progress and a number of potent functions of these two mast cell proteases were found in last decade. These include induction of microvascular leakage in skin of guinea pig[26], stimulation of inflammatory cell accumulation in peritoneum of mouse[27,28] and modulation of mast cell degranulation[29,30], indicating that they may be key mediators of allergic inflammation and promising targets for diagnosis and therapeutic intervention[31-35]. However, little is known about the function of hMC-CP except for its ability to cleave angiotension I[13]. This could be resulted from lack of sufficient amount of hMC-CP. In the current study, a procedure for cloning and expression of hMC-CP was developed and enzymatically active human intestinal recombinant MC-CP was produced.

MATERIALS AND METHODS

Materials

RNA extract kit, total RNA purification system, multi-copy *Pichia* expression kit were purchased from Invitrogen (Carlsbad, CA, USA). First strand cDNA synthesis kit, restriction endonucleases, T_4 DNA ligase, the expression vector pMAL-c2x, *E.coli* hosts TB1 and amylose resin were obtained from Biolabs (Beverly, MA,USA). Antibiotics, isopropyl thio-β-D-galctopyranoside (IPTG), heparin agarose, extr-Avidin peroxidase, biotinlated sheep anti-mouse IgG, for *E.coli* growth medium and *P. Pichia* growth medium were from Sigma (Saint Louis, MO, USA). Qiaquick gel extraction kit and *Taq* polymerase were from Qiagen (Hilden, Germany). Protein molecular weight markers were from Bio-Rad (Hercules, CA, USA). A monoclonal antibody against human mast cell carboxypeptidase (CA5) was donated by University of Southampton, UK. All other chemicals were of analytical grade.

Tissue preparation

Human colon tissue was obtained from patients with carcinoma

of colon at colectomy. Only macroscopically normal tissue was used for the study. The specimens were kept in liquid nitrogen until use.

Extraction of RNA

Total cellular RNA was extracted from normal colon tissues according to the manufacturer' s protocol. The purity of RNA was confirmed by formaldehyde denaturing agarose gel electrophoresis, and the concentration of RNA was determined with a spectrophotometer (DU640, Beckman).

Synthsis of cDNA

cDNAs were generated from total RNA by using the ProtoScript™ first strand cDNA synthesis kit. A total of 10 μL of RNA (1 μg), 2 μL of oligo (dT) primer and 4 μL of 2.5 mM dNTP were heated at 70 ℃ for 5 min. Reverse transcription was performed for 1 h at 42 ℃ in a solution (20 μL of total volume) containing 1 μL of 25U/μL M-MuLV reverse transcriptase. The reaction was terminated by incubating the mixture at 95 ℃ for 5 min, and placed on ice immediately.

PCR amplification and cloning of cDNAs

Based on the published DNA sequence of human skin mast cell carboxypeptidase[22], a pair of primers (P1: 5' -GCTATGAGGCTCATCCTGCCTGT-3' ; P2: 5' -GCTTTAGGAAGTATGCTTGAGGATATAC-3') were used to amplify hMC-CP cDNA. A hot-star PCR protocol was followed under the condition: at 95 ℃ for 15 min prior to amplification, then at 94 ℃ for 30 s, at 57 ℃ for 30 s, and at 72 ℃ for 1 min. The amplification was carried out for 30 cycles, followed by incubation at 72 ℃ for 10 min. The PCR products were analyzed with 1% agarose gel electrophoresis, and recovered with a Qiaquick gel extraction kit. The purified PCR product was cloned to pGEM-T Easy vector, forming a new plasmid pGEM/hMC-CP. The ligation mixtures were transformed into *E.coli* Dh5α. The positive recombinant clones were seeded on LB/agar plates with 100 μg/mL ampicillin, and the clones were further determined by PCR and DNA sequencing using a DNA sequencer (ABI 377 PRISM).

Construction of expression vector

To express hMC-CP in *E. coli*, an expression plasmid comprising the expression vector pMAL-C2x and *hMC-CP* cDNA was constructed. For this construction, a pair of specific primers (P3: 5' -GCTGAATTCATCGAGGGAAGGATCCCAGGC AGGCACAGCTAC-3' ; P4: -GCTCTGCAGTTAGGAAGT ATGCTTGAGGATATAC-3') were designed and used to amplify the coding region of the mature hMC-CP. The forward primer contained the recognition sequences for *Eco*R I, coding sequences for Factor Xa rEcognition sequence and the N-terminal region of the mature hMC-CP, and the reverse primer contained the rEcognition sequences for *Pst* I, and the coding sequence of the C-terminal region of the mature hMC-CP. pGEM/hMC-CP was used as the template for PCR. The resulting PCR fragments and pMAL-c2x plasmid were digested with *Eco*R I and *Pst* I. The fragments of interests were recovered from agarose gel, purified and ligated by T4 DNA ligase, which resulted in the expression plasmid pMAL/hMC-CP. The ligation mixtures were used to transform *E.coli* TB1 cells. The positive recombinant products were selected on LB agar plates with 100 μg/mL ampicillin, and confirmed by PCR and DNA sequencing.

To express hMC-CP in pichia pastoris, aNother expression plasmid comprising the expression vector pPIC9K and hMC-CP cDNA was constructed. For this construction, a pair of specific primers (P5: 5' -GCTGAATTCATCCCAGGCAG GCACAGCTAC-3' ; P6: -TACGCGGCCGCTTAGGAAG TATGCTTGAGGATATAC-3') were designed and used to amplify the coding region of the mature hMC-CP. The forward primer contained the recognition sequences for *Eco*R I, and the reverse primer contained the recognition sequences for *Pst* I. pGEM/hMC-CP was used as the template for PCR. The resulting PCR fragments and pPIC9K plasmid were digested with *Eco*R I and *Not* I. The fragments of interests were recovered from agarose gel, purified and ligated by T4 DNA ligase, which resulted in the expression plasmid pPIC9K/hMC-CP. The ligation mixtures were used to transform *E.coli* DH5α cells. The positive recombinant products were selected on LB agar plates with 100 μg/mL ampicillin. The nucleotide sequences of cDNA insert and flanking sequence were verified. The expression plasmid pPIC9K/hMC-CP was linearized by digestion with *Bgl* II. Competent cells of *P. pastoris* GS115 were prepared for electroporation with the linearized plasmid pPIC9K/hMC-CP. The electroporation was performed in a 2 mm gap cuvette at 2.0 kV, 25 μF, and 200Ω using a gene-pulser (Bio-Rad). Transformants were screened for a His^+ pheNotype on minimal dextrose (MD) agar plates. MD and minimal methanol (MM) plates were used to identify Mut^S clones. YPD plates containing Geneticin at a final concentration of 0.25, 0.5, 0.75, 2.0, 3.0, 4.0 mg/mL were used to screen multiple inserts for further expression.

Expression of recombinant hMC-CP

E.coli TB1 cells harboring the expression plasmid pMAL/hMC-CP were inoculated into LB medium containing 100 μg/mL ampicillin overnight at 37 ℃ in an orbital shaker (220 rpm). IPTG was added to a final concentration of 0.3 mM before the culture mixture was transferred to a 23 ℃ air shaker.

For the expression of hMC-CP in *P. pastoris*, a single colony of GS115 harboring the expression plasmid pPIC9K/hMC-CP was inoculated into 200 mL of buffered minimal glycerol complex medium (BMGY), and grew at 30 ℃ until the culture reached an A_{600}=2.0. Cultured cells were harvested by centrifugation and transferred to 1/10 of the original culture volume of buffered minimal methanol complex medium (BMMY), then grew at 30 ℃. Methanol was added to a final concentration of 0.5% (v/v) every 24 h to maintain induction.

Purification of recombinant hMC-CP

At 24 h after induction, the bacterial cells were harvested by centrifugation at 5 000 g for 10 min at 4 ℃. The pellet was resuspended in 50 mL ice-cold cells lysis buffer (20 mM Tris, 200 mM NaCl, 0.01% Triton X-100) at pH 8.0, then sonicated 6 times for 10 s (300 w) at 30 s intervals. The clarified cell extract was obtained by centrifugation at 20 000 g for 20 min, at 4 ℃.

Amylose resin was used for purification of the fusion protein, and equilibrated with the running buffer (20 mM Tris, 200 mM NaCl, pH8.0), then the cell extract was loaded onto the column at a flow rate. The fusion protein was eluted from column with a buffer containing 10 mM maltose. In order to obtain the recombinant hMC-CP, the fusion protein was cleaved with factor Xa in 20 mM Tris, pH 8.0, containing 100 mM NaCl, 2 mM $CaCl_2$. The digestion was performed at 23 ℃ for 3 h. The above cleavage mixture was applied to heparin agarose in an equilibration buffer (20 mM Tris, 200 mM NaCl, pH8.0), and eluted from heparin agarose by the elution buffer containing 20 mM Tris, 2 M NaCl, pH 8. The fractions containing hMC-CP were collected and stored at -80 ℃. The procedures above were mainly performed at 4 ℃.

SDS-PAGE and Western blotting analysis

SDS-PAGE was performed on a 15% polyacrylamide gel. The gel was then stained with 0.25% Coomassie brilliant blue R-250

or transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting. The membranes were incubated for 1 h at room temperature in PBS containing 4% BSA and 0.02% Tween-20 in order to prevent nonspecific binding. After incubated with CA5, biotinylated sheep anti-mouse antibody followed by extr-avidin peroxidase was added to the strips. The immunoreactive protein was visualized by DAB.

N-terminal amino acid sequence analysis

Protein was sequenced by automated Edman degradation on a model 491A protein sequencer (Applied Biosystem). Purified protein was applied to a SDS-PAGE. After blotting, the polyvinylidene difluoride membranes were stained with Coomassie brilliant blue R-250, the protein bands of interest were cut out for N-terminal amino acids sequence determination.

Protein assay

Protein concentration was determined using the method of Bradford with the protein assay dye reagent concentrator (Bio-Rad) and bovine serum albumin (BSA) was used as a standard protein.

Enzyme activity assay

In this study, the hMC-CP activity was measured spectrophometrically by hydrolyzing a substrate of synthesis peptide of hippuryl-L-phenylalanine[13]. The rate of hydrolysis of hippuryl-L-phenylalanine was determined by measuring the increase in absorbance at 254 nm. The assay mixtures contained 1 mM substrate in 0.05 M Tris-HCl, pH 7.5, 0.5 M NaCl. $\triangle A_{254}$/minute from the initial linear portion of the curve was determined. Unit definition: One unit hydrolyzes one micromole of hippuryl-L-phenylalanine per minute at pH 7.5 and 25 ℃. Bovine pancreatic CPA (51 U/mg, Sigma) was used as positive control.

RESULTS

RT-PCR amplification of hMC-CP cDNA

RT-PCR was performed with total RNA template extracted from human colon tissues. The PCR product showed a single band about 1 250 bp on 1% agarose gel (Figure 1A).

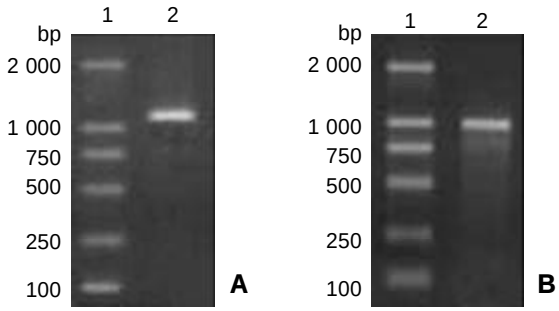

Figure 1 Agarose gel electrophoresis of PCR product. A: hMC-CP cDNA (lane 1: DNA molecular marker; lane 2: hMC-CP cDNA). B: coding region of mature human colon MC-CP cDNA (lane 1: DNA molecular marker; lane 2: PCR product of colon MC-CP cDNA).

DNA sequencing revealed that the human colon MC-CP cDNA had 1 254 bp. The DNA sequence and deduced amino acid sequence of human colon MC-CP are shown in Figure 2. The DNA sequence of human colon MC-CP cDNA was identical to the skin MC-CP except for five nucleotides at the positions 109, 575, 576, 708, 737: C→G, C→T, T→C, A→C, A→T (nucleotide numbering starts from the first codon of mature enzyme). Only the fourth (A1035→C) of the nucleotide differences described above did Not represent an altered amino acid residue in the putative protein products. The other 4

```
1   ATC CCA GGC AGG CAC AGC TAC GCA AAA TAC AAT AAT TGG GAA AAG ATT GTG GCT TGG ACT
    I   P   G   R   H   S   Y   A   K   Y   N   N   W   E   K   I   V   A   W   T
61  GAA AAG ATG ATG GAT AAG TAT CCT GAA ATG GTC TCT CGT ATT AAA ATT GGA TCT ACT GTT
    E   K   M   M   D   K   Y   P   E   M   V   S   R   I   K   I   G   S   T   V
121 GAA GAT AAT CCA CTA TAT GTT CTG AAG ATT GGG GAA AAG AAT GAA AGA AGA AAG GCT ATT
    E   D   N   P   L   Y   V   L   K   I   G   E   K   N   E   R   R   K   A   I
181 TTT ATG GAT TGT GGC ATT CAC GCA CGA GAA TGG GTC TCC CCA GCA TTC TGC CAG TGG TTT
    F   M   D   C   G   I   H   A   R   E   W   V   S   P   A   F   C   Q   W   F
241 GTC TAT CAG GCA ACC AAA ACT TAT GGG AGA AAC AAA ATT ATG ACC AAA CTC TTG GAC CGA
    V   Y   Q   A   T   K   T   Y   G   R   N   K   I   M   T   K   L   L   D   R
301 ATG AAT TTT TAC ATT CTT CCT GTG TTC AAT GTT GAT GGA TAT ATT TGG TCA TGG ACA AAG
    M   N   F   Y   I   L   P   V   F   N   V   D   G   Y   I   W   S   W   T   K
361 AAC CGC ATG TGG AGA AAA AAT CGT TCC AAG AAC CAA AAC TCC AAA TGC ATC GGC ACT GAC
    N   R   M   W   R   K   N   R   S   K   N   Q   N   S   K   C   I   G   T   D
421 CTC AAC AGG AAT TTT AAT GCT TCA TGG AAC TCC ATT CCT AAC ACC AAT GAC CCA TGT GCA
    L   N   R   N   F   N   A   S   W   N   S   I   P   N   T   N   D   P   C   A
481 GAT AAC TAT CGG GGC TCT GCA CCA GAG TCC GAG AAA GAG ACG AAA GCT GTC ACT AAT TTC
    D   N   Y   R   G   S   A   P   E   S   E   K   E   T   K   A   V   T   N   F
541 ATT AGA AGC CAC CTG AAT GAA ATC AAG GTT TAC ATC ACC TTC CAT TCC TAC TCC CAG ATG
    I   R   S   H   L   N   E   I   K   V   Y   I   T   F   H   S   Y   S   Q   M
601 CTA TTG TTT CCC TAT GGA TAT ACA TCA AAA CTG CCA CCT AAC CAT GAG GAC TTG GCC AAA
    L   L   F   P   Y   G   Y   T   S   K   L   P   P   N   H   E   D   L   A   K
661 GTT GCA AAG ATT GGC ACT GAT GTT CTA TCA ACT CGA TAT GAA ACC CGC TAC ATC TAT GGC
    V   A   K   I   G   T   D   V   L   S   T   R   Y   E   T   R   Y   I   Y   G
721 CCA ATA GAA TCA ACA ATT TAC CCG ATA TCA GGT TCT TCT TTA GAC TGG GCT TAT GAC CTG
    P   I   E   S   T   I   Y   P   I   S   G   S   S   L   D   W   A   Y   D   L
781 GGC ATC AAA CAC ACA TTT GCC TTT GAG CTC CGA GAT AAA GGC AAA TTT GGT TTT CTC CTT
    G   I   K   H   T   F   A   F   E   L   R   D   K   G   K   F   G   F   L   L
841 CCA GAA TCC CGG ATA AAG CCA ACG TGC AGA GAG ACC ATG CTA GCT GTC AAA TTT ATT GCC
    P   E   S   R   I   K   P   T   C   R   E   T   M   L   A   V   K   F   I   A
901 AAG TAT ATC CTC AAG CAT ACT TCC TAA
    K   Y   I   L   K   H   T   S  stop
```

Figure 2 Nucleotide sequence and deduced amino acid sequences of mature human colon MC-CP. The nucleotide variations and amino acid substitutions different from the skin MC-CP are underlined and boxed, respectly.

nucleotide variations caused 3 amino acid substitutions at the positions 146, 301, 355. The human colon MC-CP had Gly^{146}, Ile^{301} and Ile^{355} residues, whereas the skin MC-CP had Arg^{146}, Thr^{146} and Asn^{355} residues. In contrast, the colon MC-CP cDNA was 100% identical to the lung MC-CP cDNA.

Construction of expression vector

A 924 bp of PCR product was obtained following amplification of the coding region of the mature hMC-CP (Figure 1B). DNA sequencing showed that the recombinant pMAL/hMC-CP and pPIC9K/hMC-CP plasmids had the correct open reading frame coding for 308 amino acids mature polypeptide and no substitutions were introduced by PCR.

Expression of recombinant hMC-CP in E.coli

As shown in Figure 3A, a high level of expression of an induced protein of about 80 kD was achieved after the *E.coli* harbouring expression plasmid pMAL/hMC-CP, which was in agreement with the expected molecular mass of the fusion protein MBP (45 kDa) and hMC-CP (36 kDa). Figure 3B showed a band at about 80 kDa (expected in *E.coli* cells with IPTG induction) reacted to CA5, suggesting that the recombinant protein had a good immunological activity. The best expression of the recombinant protein after IPTG induction was at 23 ℃ for 16 h (Figure 4). The recombinant products generated by the above procedures were mainly insoluble inclusion body with a small proportion of the soluble recombinant proteins (Figure 5).

In *P. pastoris* expression, 2 colonies resistant to 4.0 mg/ml Geneticin were screened and used for the expression of recombinant protein. There was a substantial quantity of recombinant proteins in cell-free supernatant, and SDS-PAGE showed a major band of approximately 37 kDa (Figure 6A), which reacted to CA5 on Western blot (Figure 6B).

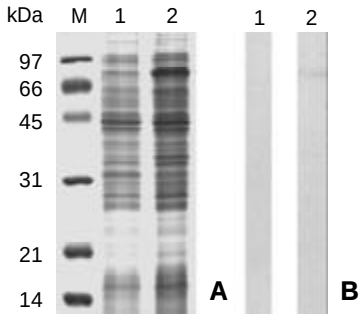

Figure 3 Analysis of recombinant proteins expressed in *E.coli*. A: SDS-PAGE. B: Western blots. M: molecular mass markers; lane 1: without IPTG induction; lane 2: with IPTG induction.

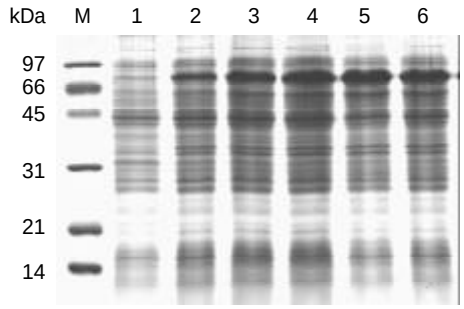

Figure 4 SDS-PAGE analysis of time course of recombinant proteins expressed in *E.coli*. lane 1: before induction; lane 2: 8 h after induction; lane 3: 16 h after induction; lane 4: 24 h after induction; lane 5: 32 h after induction.

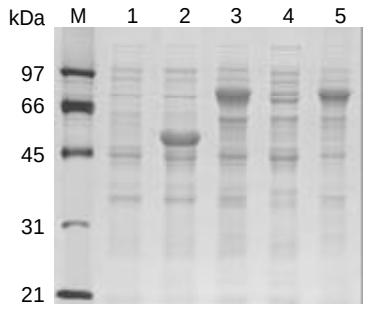

Figure 5 SDS-PAGE analysis of rhMC-CP expressed in *E. coli* TB1 cells. M: molecular weight markers; lane 1: total cellular protein of *E.coli* TB1 cells without IPTG induction; lane 2: total cellular protein of *E.coli* TB1 cells with IPTG induction (control vector); lane 3: total cellular protein of *E.coli* TB1 cells with IPTG induction; lane 4: soluble fraction of cell lysate from *E.coli* TB1 with IPTG induction; lane 5: precipitated fraction of cell lysate from *E.coli* TB1 with IPTG induction.

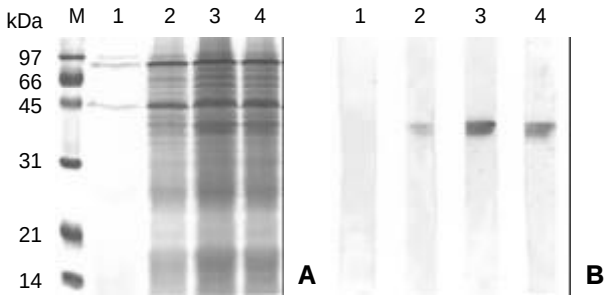

Figure 6 Induction of recombinant HMC-CP expressed in *P. pastoris*. A: secreted proteins analyzed by SDS-PAGE. B: Western blot analysis of secreted proteins with HMC-specific monoclonal antibody, clone CA5. M: molecular weight markers; lane 1: 0 h; lane 2: 24 h; lane 3: 48 h; lane 4: 72 h.

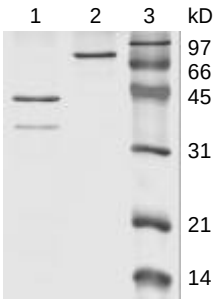

Figure 7 SDS-PAGE analysis of fusion protein cleavage (lane 1: cleaved by factor Xa; lane 2: uncleaved by factor Xa; lane 3: molecular weight marker).

Purification of rhMC-CP

Maltose-binding protein (MBP) was used as a fusion partner to provide a "tag" which could be used for the subsequent purification. The yield of the recombinant fusion protein was 12 mg/L of bacterial culture. The purified fusion protein showed a single protein band of approximately 80 kDa on SDS-PAGE.

After the fusion protein cleavage, SDS-PAGE analysis showed that the fusion protein was completely cleaved by factor Xa (Figure 7). The cleavage mixtures were loaded to heparin agarose, and the target protein showed one band about 36 kDa on SDS-PAGE, which was corresponding to the molecular weight of the native hMC-CP published previously (Figure 8A). About 1.2 mg pure recombinant protein was obtained from 5 mg fusion protein following the above procedures. The Western blot showed that this 36 kDa protein band strongly

reacted to CA5 (Figure 8B), suggesting that the recombinant protein had a good immunology activity. The N-terminal sequence of the purified recombinant protein expressed in *E.coli* was IPGRHSYAKY, and no additional amino acids were found at the N-terminus.

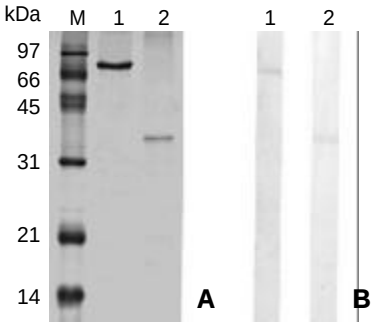

Figure 8 Analysis of purified recombinant protein. A: SDS-PAGE analysis of purified recombinant protein. B: Western blot analysis of purified fusion protein with CA5. Lane 1: purified fusion protein after MBP affinity chromatography; lane 2: purified recombinant hMC-CP after heparin agarose affinity chromatography.

Analysis of enzymatic activity

The purified recombinant hMC-CP expressed in *E.coli* had a very low level of enzymatic activity. In contrast, enzymatic activity in cell-free supernatant of *P. pastoris* culture was 11.7 U/mg secreted protein.

DISCUSSION

A cDNA of human colon hMC-CP was cloned and active enzyme was expressed in the current study, which will offer an essential tool for investigating the functions of hMC-CP, a zinc containing metalloexopeptidase.

Our result revealed that the human colon MC-CP cDNA comprised 1 251 bp, which agreed with the skin and lung mast cell carboxypeptidase[22,23,26]. The hMC-CP was predicted to be translated as a 417 amio acid preproenzyme which includes a 15 amino acid signal peptide, a 94 amino acid activation peptide and 308 amino acid mature mast cell carboxypeptidase. When comparison of the DNA sequence of human colon MC-CP cDNA with skin MC-CP cDNA, five variations were found which caused 3 amino acid substitutions, but there was Not any difference between the human colon and skin MC-CP. The meaning of these variations between tissues in man requires more investigations.

Since the role of hMC-CP in man remains unclear and human mast cells contain large amount of MC-CP, there is a pressing need to investigate the functions of this enzyme. One of the difficulties in investigating the potential functions of MC-CP over the years was that it was uneasy to obtain a substantial quantity of the active enzyme. Purification of MC-CP from human tissues was not only hard to perform, but also difficult to collect enough tissues for purification. Therefore, development of an efficient heterologous expression system for the production of recombinant hMC-CP is an alternative for obtaining a sufficient quantity of hMC-CP. There are a number of options for heterologous recombinant expressions, among them *E.coli* expression system is the most convenient and frequently used, therefore, *E.coli* expression system was used to express hMC-CP. The pMAL-C2x plasmid[36,37], a vector that allows the fusion of the target protein N-terminus to the MBP tag, made the purification of recombinant proteins much easier.

The extra residue(s) is often added to the C-terminus or N-terminus of recombinant protein. In this study, a pair of specific primers were designed. The upstream primer contained the sequences for a factor Xa recognition site just before the sequence for N-terminus of hMC-CP, and the downstream primer contained a terminator. The PCR product was inserted into the expression vector pMAL-c2x, which yielded the recombinant protein without extra residues after the fusion protein was cut with factor Xa. The result of N-terminal amino acid sequencing also showed that the N-terminus of recombinant hMC-CP had no extra residue.

After induction with IPTG, the recombinant protein was expressed in *E.coli*, with a molecular weight of about 80 kDa. This was in agreement with the expected molecular mass of the fusion proteins MBP (45 kD) and HMC-CP (36 kD). It was reported that the target gene fused to bacterial gene could improve the expression level and increase the solubility of recombinant proteins[37]. The expression vector pMAL-2x containing *malE* gene of *E.coli* encoding MBP was used for fusion expression. The target gene was inserted downstream from the *malE* gene, which resulted in the expression of hMC-CP fused to MBP. The solubility of recombinant proteins generally could be increased when the cell culture temperature decreased[38]. In our case, although the culture temperature was reduced to 23 ℃, insoluble recombinant proteins were still the major products. Since purification of recombinant proteins from inclusion body was a complicated process, we only used soluble products to isolate active recombinant hMC-CP.

Fusion protein was purified with one–step affinity chromatography with maltose. Once the fusion protein was isolated, it was necessary to remove the tag. In this study, the linker sequence recognized by factor Xa was designed between the MBP and target protein, because there were no such sequences in MBP and hMC-CP. After the fusion protein cleavage, usually ion exchange chromatography and hydroxyapatite chromatography were used in separating the protein of interest from MBP[36,39]. But in this study, the recombinant protein was purified by heparin agarose chromatography as MC-MBP could tightly bind to heparin[40]. In comparison with ion exchange chromatography and hydroxyapatite chromatography, heparin agarose chromatography was simpler and more convenient. Approximately1.2 mg target protein was obtained from 5 mg fusion protein following the established procedures. N-terminal amino acid sequencing showed that the first 10 amino acids of the recombinant hMC-CP were in good agreement with the human skin and lung MC-CP. Western blotting analysis showed that the recombinant protein had the similar immuno-reactivity with its natural counterpart, indicating that the recombinant hMC-CP could be used as an antigen to produce a specific antibody.

Our studies revealed that the purified recombinant hMC-CP expressed in *E.coli* had a very low level of enzymatic activity to substrate hippuryl-L-phenylalanine. It might be possible that the *E. coli* expression system is a prokaryotic expression system, which can not carry out post-translation modifications. In order to obtain higher levels of enzymatic activity of recombinant hMC-CP, we used *P. pastoris* to express hMC-CP. The enzymatic assay showed that the hMC-CP expressed in *P.asptoris* had a relatively high activity (11.7 U/mg secreted protein) towards hippuryl-L-phenylalanine. It is possible that *P.asptoris* is an eukaryotic expression system, which has the ability to perform eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing[41]. Our result showed that the supernatant of *P.pastoris* culture had the highest enzymatic activity on the second day after induction by methanol, the enzymatic activity would decrease when induction time increased. It is possible that the secreted recombinant protein was degraded with the increase of induction time.

In conclusion, cDNA encoding human colon MC-CP can

be cloned and expressed in *E.coli* and *P.asptoris*. The expression of recombinant hMC-CP can facilitate its functional study including its role in intestinal diseases.

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• GASTRIC CANCER •

Apoptosis of human gastric adenocarcinoma cells induced by b-ionone

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Abstract

AIM: To investigate the effect of β-ionone on the growth and apoptosis of gastric adenocarcinoma cell line SGC-7901.

METHODS: Using MTT, fluorescence dye (Hoechst-33258), transmission electron microscopy and the TUNEL assay, we examined growth and apoptosis of SGC-7901 cells treated with β-ionone at various concentrations (*i.e.* 25, 50, 100 and 200 μmol/L) for 24 h, 48 h.

RESULTS: The growth of SGC-7901 cells was inhibited by β-ionone. Seven days after treatment with β-ionone at four concentrations, the inhibition rates were 12.04%, 30.59%, 78.25% and 94.15%, respectively. The IC_{50} value of β-ionone for SGC-7901 cells was estimated to be 89 μmol/L. The apoptotic morphology was demonstrated in SGC-7901 cells treated with β-ionone by Hoechst-33258 staining and electron microscopy. Apoptosis was also shown in β-ionone-treated SGC-7901 cells by the TUNEL assay.

CONCLUSION: β-ionone can inhibit cell proliferation and induce apoptosis of SGC-7901 cells. However, the mechanism needs to be further investigated.

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INTRODUCTION

Following the dietary guidelines[1,2] that emphasize the importance of fruits, vegetables and grains could lead to an estimated 35% reduction in cancer deaths[3], fruits, vegetables and grains are potential sources of anticarcinogenic agents[4-11], such as nondigestible carbohydrates and micronutrients including β-ionone, ascorbic acid, γ-tocopherol, isoprenoids and folic acid. Epidemiological evidence suggested that intake of these constituents could lead to a reduced risk of chronic diseases such as cancer and cardiovascular disorders[3,12-14]. β-ionone, enriched in many fruits, vegetables and grains, is a common intermediate product of up to 22 000 isoprenoids[15] and could exert potent anticarcinogenic and antitumor activities at the pharmacological level[16,17]. It has been documented that β-ionone could suppress the proliferation of melanoma, breast cancer and meningioma cells[18-21]. β-ionone has also been shown to have an effect on apoptosis of vascular endothelial cells[22] and tumor cells[23,24]. Although its exact mechanisms are not clear, the inhibitory effect of β-ionone on the proliferation of rapidly dividing cells has been demonstrated. In many instances, growth inhibition following terminal differentiation or anticancer drug treatment results in apoptosis.

Gastric cancer is one of the most frequent neoplasms in China and some other parts of the world[25-33]. Chemoprevention is always used as the main treatment for advanced cancer, and has been under intense investigation[34-49]. Understanding the mechanisms through which β-ionone suppresses the proliferation of gastric adenocarcinoma cells could provide a way to prevent this disease in general population. Therefore, in this study, we explored whether and how β-ionone induced apoptosis of human gastric adenocarcinoma cells.

MATERIALS AND METHODS

b-ionone

β-ionone, 4-(2, 6, 6-trimethyl-1-cyclohexenyl)-3-buten-2-one with >95 % purity, was purchased from Sigma (USA). It was dissolved in absolute ethanol and diluted to the following concentrations: 25, 50, 100 and 200 μmol/L.

Cell culture

Human gastric adenocarcinoma cell line, SGC-7901 (Cancer Research Institute of Beijing, China), was cultured in RPMI 1640 medium (pH 7.2-7.4), supplemented with fetal calf serum 10%, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 ℃ with a humidified atmosphere of 5% CO_2. The cells were sub-cultured with a mixture of ethylenedinitrile tetraacetic acid (EDTA) and trypsin (Sigma).

MTT assay for determination of cell growth

SGC-7901 cells were seeded in eight 96-well plates, each well containing 3.0×10^3 cells. After 24 h, different concentrations of β-ionone were added with. On the next day, 0.05 mL of 1.0 mg/ml 3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide (MTT, Sigma) was added to one of the eight plates. After 4 h, culture media were discarded followed by addition of 0.2 ml dimethyl sulfoxide (DMSO) and vibration for 2 min. The absorbance was measured at 570 nm using a model 550 microplate Reader. β-ionone concentration required to inhibit the net increase in the 48 h cell count by 50 % (IC_{50}) was calculated based on the plots of data from three or more evaluations. The means were obtained on each of seven days and used to draw a curve of cell proliferation. The inhibitory rates (IR) on the 7th day were calculated as follows:

$$IR(\%)=\frac{\text{Total absorbance in negative control (7 d)} - \text{Total absorbance in the tested group (7 d)}}{\text{Total absorbance in negative control (7 d)}}\times100\%$$

Hoechst-33258 staining for determination of morphological changes of apoptotic cells

Morphological changes of apoptotic cells were determined by fluorescence microscopy. Briefly, the cells were collected and fixed in 100% ethanol, stained with 0.5 mmol/L Hoechst-33258 (Sigma) for 3 min at 37 ℃, then visualized under a fluorescence microscope with ultraviolet (UV) excitation at 300-500 nm. The cells with nuclei containing condensed chromatin or cells with fragmented nuclei were defined as apoptotic cells.

Observation of apoptotic cells with transmission electron microscopy

The cultured SGC-7901 cells treated with β-ionone at different concentrations were scraped off and harvested. Subsequently, the cells were immersed with Epon 821, embedded in capsules and converged at 60 ℃ for 72 h, and then ultrathin sections (60 nm) were prepared and stained with uranyl acetate and lead citrate. The ultrastructure of cells was examined by transmission electron microscopy.

TUNEL assay for determination of apoptosis cells

The TUNEL reaction could preferentially label DNA strand breaks generated during apoptosis, and allow discrimination of apoptosis from necrosis and primary DNA strand breaks induced by cancer chemoprevention agents. SGC-7901 cells were treated for 24 h and 48 h with β-ionone at various concentrations and collected by centrifugation. Specimens were fixed immediately in 4% formaldehydum polymerisatum and embedded in paraffin. Apoptosis of SGC-7901 cells was evaluated by using an *in situ* cell death detection kit (Roche Co. Ltd, Germany). The sections were deparaffinized in xylene and rehydrated through graded alcohol, treated with proteinase K and then 0.3% H_2O_2, labeled with fluorescein dUTP in a humidified box for 1 h at 37 ℃. The cells were then combined with POD-horseradish peroxidase, colorized with diaminobenzidine tetrahydrochloride (DAB). Controls consisted of omission of fluorescein dUTP. Cells were visualized under a light microscope. The apoptotic index (AI) was calculated as follows: AI=(number of apoptotic cells/total number counted)×100%.

Statistical analysis

Data were expressed as mean±SD. Analysis of data was performed using the Student's *t* test or one-way ANOVA. $P<0.05$ was considered as statistically significant.

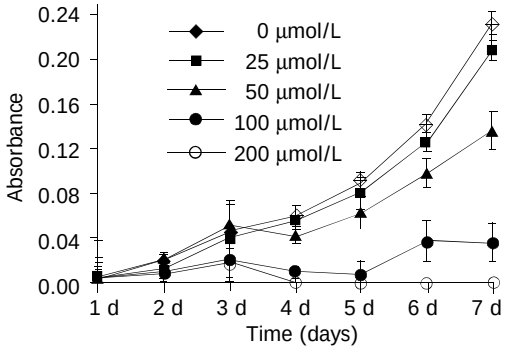

Figure 1 Proliferation kinetics of SGC-7901 cells treated with β-ionone at various concentrations.

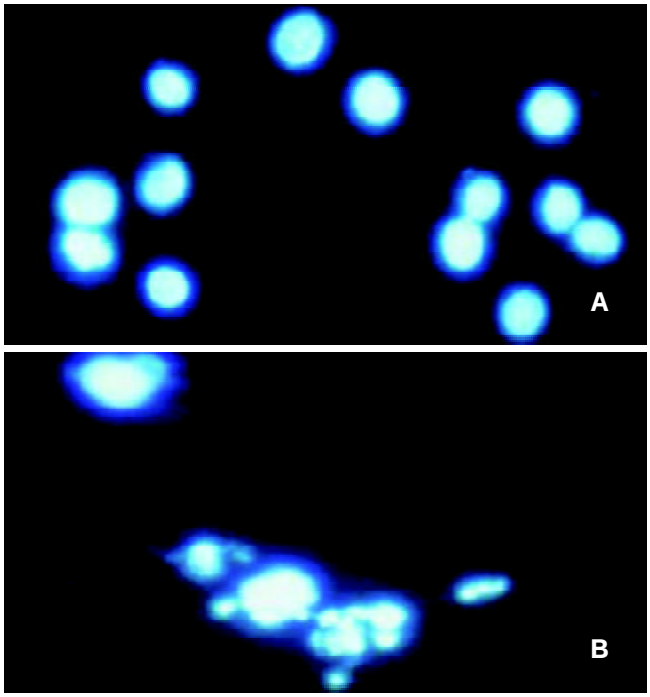

Figure 2 β-ionone-induced apoptosis in SGC-7901 cells stained with Hoechst-33258 (×400). A: control SGC-7901 cells, B: experimental cells treated with β-ionone showing nuclear shrinkage or fragmentation.

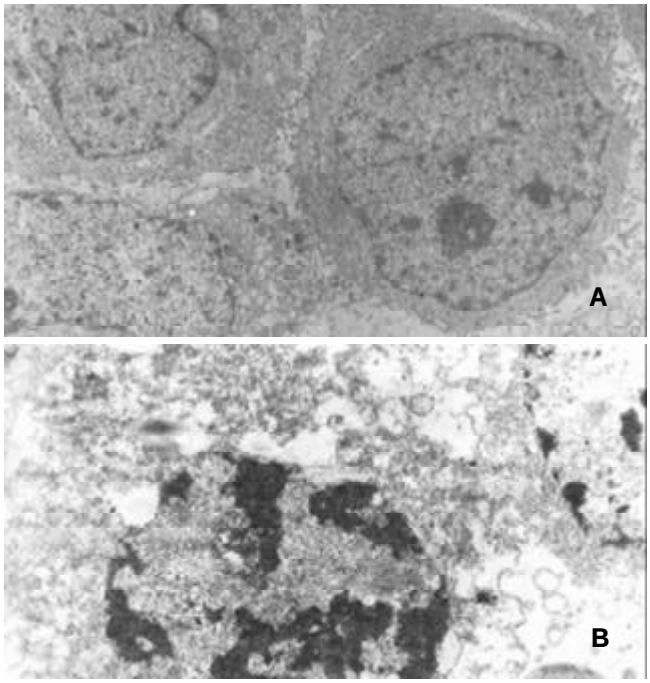

Figure 3 Ultrastructural changes of SGC-7901 cells treated with 100 μmol/L β-ionone for 24 h (×15000). A: SGC-7901 cells in the control group, B: experimental SGC-7901 cells treated with β-ionone showing early changes of apoptosis in which nuclear chromatin condensation and cell shrinkage were observed.

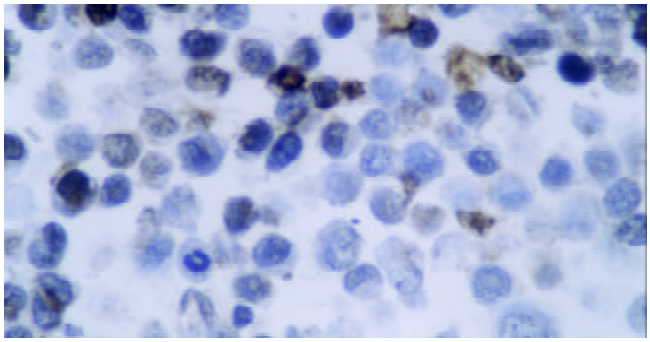

Figure 4 Apoptotic cells induced by β-ionone (200 μmol/L 48 h) in SGC-7901 cells detected TUNEL assay (×400).

RESULTS

Inhibition of SGC-7901 cell proliferation by b-ionone

SGC-7901 cells were treated with β-ionone at various concentrations for 1-7 days, and the cell viability was determined as described above by MTT assay. As shown in Figure 1, β-ionone inhibited the growth of gastric adenocarcinoma cells in a dose- and time-dependent manner. The growth of the cells treated with β-ionone at concentrations of 100 and 200 μmol/L was significantly slower than that of the negative control within 7 days (P<0.05). SGC-7901 cells incubated with 25 and 50 μmol/L of β-ionone grew slower, albeit not significantly than the negative control. β-ionone at 100 μmol/L and 200 μmol/L had an inhibitory effect of more than 90% on tumor cell growth 3 days after treatment. The inhibitory rate at the four progressively increasing concentrations of β-ionone was 12.04%, 30.59%, 78.25% and 94.15% 7 days after treatment. The IC_{50} value for β-ionone was estimated to be 89 μmol/L.

Morphological changes of apoptotic SGC-7901 cells treated with b-ionone

SGC-7901 cells in the negative control group exhibited a round shape, clear edge and homogeneous staining (Figure 2A). However, after 24 h and 48 h exposure to β-ionone, SGC-7901 cells began to show morphologic features of apoptosis, such as cell shrinkage, chromatin condensation and nuclear pyknosis (Figure 2B). The ultrastructural changes were observed by electron microscopy showing early changes of apoptosis including nuclear chromatin condensation and cell shrinkage (Figures 3A & 3B).

Apoptosis detected by TUNEL assay in SGC-7901 cells treated with b-ionone

The cells with positive staining in the nuclei were defined as apoptotic cells (Figure 4). The results showed that after treatment of SGC-7901 cells with β-ionone at different concentrations for 24 h and 48 h, the AIs were significantly increased in a dose- and time-dependent manner (Table 1).

Table 1 Apoptotic index (AI) of SGC-7901 cells treated by β-ionone

β-ionone (μmol/L)	AI (%)[a]	
	24 h	48 h
0	0.15±0.087	-
25	0.12±0.03	0.87±0.23[b]
50	0.47±0.15	3.43±1.00[b]
100	1.67±0.53[b,c]	8.50±19.2[b,c]
200	4.78±1.32[b,c,d]	11.85±3.83[b,c,d]

a: X experiments were performed and Y cells were counted in each experiment, b: P<0.05, comparison between 24 h and 48 h, c: P<0.05 compared with negative control, d: P<0.05 as determined by one-way ANOVA.

DISCUSSION

Epidemiologic studies have revealed a strong inverse association between frequency of intake of plant-derived foods and cancer incidence[3]. β-ionone, widely distributed in fruits and vegetables, has been found to be a common intermediate product of up to 22 000 isoprenoids[15] and many other phytochemicals. It could exert broad and potent anticarcinogenic and antitumor activities when fed at pharmacological levels. β-ionone could also suppress the proliferation of melanoma and breast cancer cells[16-18]. However, the effects of β-ionone on gastric adenocarcinoma cells have not been reported.

Isoprenoid-mediated suppression of cell growth is clearly independent of a mutated *ras* function. β-ionone could suppress the proliferation of B16 and HL-60 cells that express ki-ras and n-ras, respectively. The study from Mo *et al*[16] showed that β-ionone also suppressed the proliferation of Caco-2 and MCF-7 cells which express wild-type ras. The action, like that of lovastatin, is independent of ras function. Both β-ionone and lovastatin can arrest HL-60 promyelocytic leukemia cells in the G_1 phase of the cell cycle in a concentration-dependent manner. The present study demonstrated that β-ionone inhibited the growth of human gastric adenocarcinoma SGC-7901 cells in a dose- and time-dependent manner. The inhibition rates were 12.04%, 30.59%, 78.25% and 94.15% 7 days after treatment with β-ionone at 25, 50, 100 and 200 μmol/L, respectively. The IC_{50} value for β-ionone was estimated to be 89 μmol/L.

Apoptosis, also known as programmed cell death, is a natural process of cell suicide which plays a critical role in the development and homeostasis of metazoans[50,51]. It is a complex process regulated by a variety of factors such as Bcl-2[52], Fas[53] and p53[54]. The isoprenoid-mediated initiation of apoptosis might be independent of a mutated p53 function. β-ionone could initiate apoptosis in B16 and MCF-7 cells which express wild type p53 and in Caco-2 cells which express mutated p53 as well as in p53-null HL-60 cells[55]. However, whether β-ionone has any effect on the other apoptotic pathways in cancer cells is not understood.

In conclusion, β-ionone can inhibit cell growth and proliferation and induce apoptosis of SGC-7901 cells. However, the mechanism by which β-ionone blocks cell proliferation and induces apoptosis of SGC-7901 cells needs to be further investigated.

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• GASTRIC CANCER •

Expression and significance of VEGF-C and FLT-4 in gastric cancer

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Abstract

AIM: To investigate the expression of pathological factors of VEGF-C and its receptor FLT-4 in primary gastric cancer and adjacent normal tissues.

METHODS: The expression of VEGF-C and FLT-4 was studied in 80 primary gastric cancers and adjacent normal tissues from the same patients by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immumohistochemistry.

RESULTS: Both primary gastric cancer and adjacent normal tissue could express VEGF-C and FLT-4, and FLT-4 expression was also detected in endothelial cells of stromal blood vessels and lymphatic vessels. There was a significant difference in expression of VEGF-C between primary tumor and adjacent normal tissue samples (P=0.01), and a statistical correlation between VEGF-C and FLT-4 expression in tumors (P=0.00886). With regard to VEGF-C expression, there was a significant difference between moderate-poor differential type and high differential type (P=0.032), and a significant difference between positive and negative lymph node metastases (P=0.024). However, there was no significant difference between positive and negative serosal invasions (P=0.219).

CONCLUSION: VEGF-C and its receptor FLT-4 play a role in the development of gastric cancer, and the tumors with expression of VEGF-C and FLT-4 are more likely to have lymph node metastasis.

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INTRODUCTION

Invasion and metastasis are the characteristics of malignant tumor. Epithelial malignancy spreads predominantly through lymphatic system, and its metastastic degree is closely related to patient' s prognosis[1]. However, very little is known about the mechanism of lymphangiogenesis and lymph node metastasis. Recent studies have shown that VEGF-C can bind to specific receptor VEGFR3/FLT-4 that express in lymphatic endothelium, stimulate lymphangiogenesis, and promote lymphatic metastasis[2-4]. We investigated the expression and significance of VEGF-C and its receptor FLT-4 in gastric cancer.

MATERIALS AND METHODS

Materials

Reagents Trizol liquid, AMV reverse transcriptase, Oligd(T)$_{14}$, RNAsin, dNTP, Taq DNA polymerase were purchased from Shanghai Sangon Biological Engineering Technology and Service Co.Ltd, PCR primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. The antibodies of VEGF-C and FLT-4 were purchased from Santa Cruz Company.

Clinical data A total of 80 patients with primary gastric cancer were analyzed, with a mean age of 53.5 years, arranging from 38 to 72 years. Routine pathological diagnosis showed that 59 cases were adenocarcinoma and 21 cases were signet carcinoma. Among them, 54 cases presented lymph node metastasis, and 26 cases had no lymph node metastasis.

Methods

Detection of expression of VEGF-C AND FLT-4 Expression of VEGF-C and FLT-4 was assessed in each gastric cancer sample and its adjacent normal tissues by semi-quantitative RT-PCR.

RNA extraction Total RNA was extracted by Trizol one-step procedure, and suspended in DEPC-treated reverse osmosis-H_20, and conserved at -70 ℃ for reverse transcription. RNA yield and purity were determined by standard UV spectrophotometric assay. The ratio of A260/A280 was 1.80.

First strand cDNA synthesis Five μg of the total RNA was dissolved in 20 μl of mixture containing 2 μl 10× first-strand buffer, 20 U AMV reverse transcriptase, 2 μl dNTP, 20 U RNAsin, 500 ng Oligd(T)$_{14}$, and DEPC-treated reverse osmosis-H_2O. The reaction conditions were as follows: at 42 ℃ for 60 min, and at 95 ℃ for 5 min. The first strand cDNA was stored at -20 ℃ until use.

PCR amplification The primers of VEGF-C, FLT-4 and β-actin were synthesized according to the primer design principles, all primers span an intron to control against amplification of genomic DNA sequences. Four μl first strand cDNA was amplified in 20 μl volume. The primers of VEGF-C yielded 206 bp product as follows: 5' -end primer: 5' -AAGGAGGCTGGCAACATAAC-3' , 3' -end primer: 5' -CCACATCTGTAGACGGACAC-3' . Following an initial denaturation at 94 ℃ for 5 min, the samples were amplified by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, extension at 72 ℃ for 30 s, and ended by extension at 72 ℃ for 10 min. The primers of FLT-4 yielded a 298 bp product as follows: 5' -end primer: 5' -AGCCATTCATCAACAAGCCT-3' , 3' -end primer: 5' -GGCAACAGCTGGATGTCATA-3' . Following an initial denaturation at 94 ℃ for 5 min, the samples were amplified by 28 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 1 min, extension at 72 ℃ for 1 min, and ended by extension at 72 ℃ for 10 min. The primers of β-actin, which was amplified with VEGF-C and FLT-4 as an internal control, yielded a 644 bp product as follows: 5' -end primer: 5' -ACGTTATGGATGATGATATCGC-3' , 3' -end primer: 5' -CTTAATGTCACGCACGATTTCC-3' . The PCR products

were separated on 1.7% agarose gel, stained with ethidium bromide, and analyzed with Quantity one 4.1.0 software. The ratios of VEGF-C/β-actin, and FLT-4/β-actin were used to semiquantify the levels of VEGF-C and FLT-4.

Immunohistochemistry Immunohistochemical studies of VEGF-C and FLT-4 expression in gastric cancer and adjacent normal tissues were performed by the avidin-biotin-peroxidase technique, as previously described[5].

Statistical analysis Statistical analyses were made with SPSS software, version 10.0.

RESULTS

Results of RT-PCR

Eighty primary gastric cancers and adjacent normal tissues from the same patients were examined for the expression of VEGF-C and FLT-4 by RT-PCR. In 80 cases of primary tumors, VEGF-C and FLT-4 were expressed in 58 (72.5%) and 51 (63.75%) cases, respectively. However, in 80 adjacent normal samples, VEGF-C and FLT-4 were expressed in 13 (16.25%) and 16 (20%), respectively. The expression of VEGF-C and FLT-4 in primary gastric cancer was significantly different from that in adjacent normal tissues (P=0.01 and P=0.038 respectively, Figure 1, Table 1).

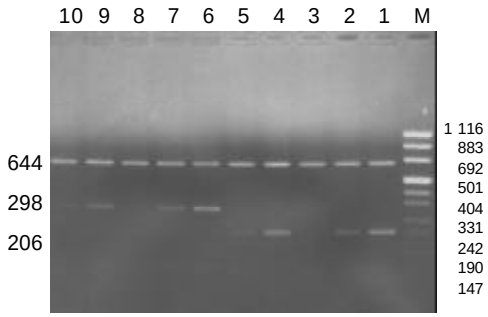

Figure 1 Semi-quantitative RT-PCR amplified products of VEGF-C and FLT-4 in primary gastric cancer and adjacent normal tissues. Lane-M: puc mix marker 8 (1 116, 883, 692, 501, 404, 331, 242, 190, 147, 110, 67, 34, 26, 19) bp, 644 bp: internal standards, 298 bp: FLT-4 expression, 206 bp: VEGF-C expression, Lane 1: Control of VEGF-C positive expression in placenta, Lane 6: Control of FLT-4 positive expression in placenta, Lanes 2,4: VEGF-C expression in primary gastric cancer, Lanes 3,5: VEGF-C expression in adjacent normal tissues, Lanes 7,9: FLT-4 expression in primary gastric cancer, Lanes 8, 10: FLT-4 expression in adjacent normal tissues.

Table 1 Expression of VEGF-C and FLT-4 in primary gastric cancers and adjacent normal tissues detected by semi-quantitative RT-PCR (mean±SD)

	Cases	VEGF-C	FLT-4
Primary gastric cancers	80	0.555±0.399[a]	0.335±0.261[b]
Adjacent normal tissues	80	0.146±0.311[c]	0.153±0.275[d]

[a]P=0.01 *vs* group c, [b]P=0.038 *vs* group d.

Meanwhile, in 58 cases of gastric cancer with VEGF-C positive expression, FLT-4 was coexpressed in 42 cases (82.35%), but 22 cases of gastric cancer with VEGF-C negative expression showed FLT-4 expression in 9 cases (40.91%). The VEGF-C expression in primary tumors was significantly correlated with FLT-4 (P=0.00886).

Result of immunohistochemistry

Human placenta served as a positive control. Positive expression of VEGF-C and FLT-4 showed brown staining in the cytoplasm of tumor or normal cells, more than 500 cells were calculated in different microscopic fields of slides, and percentage of positive cells was evaluated. Staining intensities were scored according to the following scale: - , no cells stained; +, less than 25% of cells stained; ++, 26% to 50% of cells stained; +++, 51% to 75% of cells stained; ++++, more than 75% of cells stained.

Immunohistochemical staining revealed that VEGF-C was expressed in 55 of 80 primary tumors (68.75%) and 11 of 80 adjacent normal tissues (13.75%), FLT-4 was expressed in 49 of 80 primary tumors (61.25%) and 14 of 80 adjacent normal tissues (17.5%), FLT-4 also expressed in endothelium of small blood and lymphatic vessels. There was a significant difference in the expression of VEGF-C and FLT-4 between gastric cancer and adjacent normal tissues (Table 2, Figure 2).

Table 2 Expression of VEGF-C and FLT-4 in primary gastric cancers and adjacent normal tissues detected by immumohistochemistry

Positive expression	Primary gastric cancer (cases)		Adjacent normal tissue (cases)	
	VEGF-C	FLT-4	VEGF-C	FLT-4
+	3	2	2	3
++	7	8	4	5
+++	20	18	3	3
++++	25	21	2	3

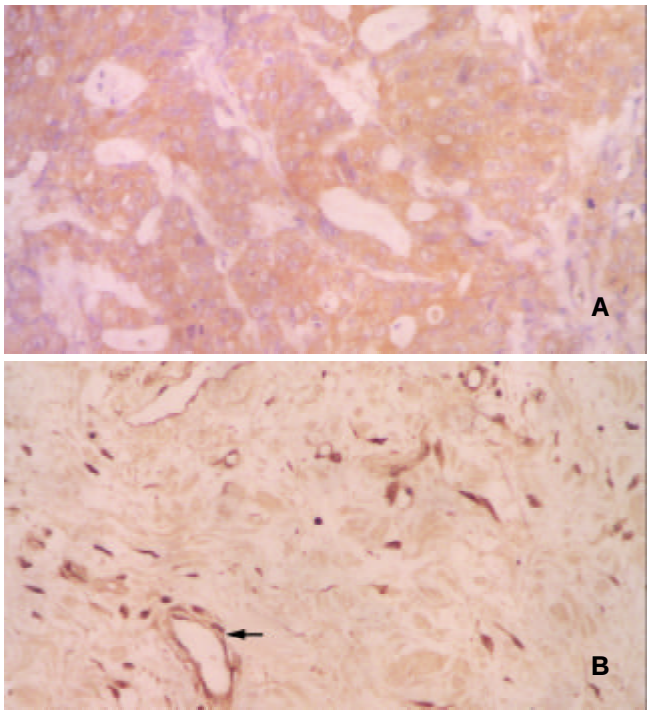

Figure 2 Expression of VEGF-C and FLT-4 in gastric cancer. A: VEGF-C positive expression in gastric cancer, ×400. B: FLT-4 positive expression in gastric cancer, ×200. (←endothelial cells of vessels).

Pathologic factors affecting expression of VEGF-C

Several pathological factors, including histological type, lymph node metastasis, serosal invasion, were investigated to predict the expression of VEGF-C in gastric cancer. The results showed that there was a significant difference in expression of VEGF-C between moderately-poorly differentiated type and highly differentiated type (P=0.032), and a significant difference between positive lymph node metastasis and negative lymph

node metastasis (P=0.024). However, there was no significant difference between positive and negative serosal invasions (P=0.219, Table 3).

Table 3 Correlation between pathological factors and VEGF-C expression in 80 primary gastric cancers

Pathological factors	No. of cases	VEGF-C mRNA (mean±SD)	P
Histological type			
Moderately-Poorly differential	51	0.485±0.345	P=0.032
Highly differential	29	0.200±0.290	
Lymph node metastasis			
Positive	54	0.579±0.290	P=0.024
Negative	26	0.282±0.314	
Serosal invasion			
Positive	59	0.567±0.268	P=0.219
Negative	21	0.390±0.330	

DISCUSSION

In the 1970' s, Folkman first found that tumor growth and metastasis were dependent on intratumoral angiogenesis. Since then, a large number of studies have emphasized the angiogenesis of tumors, but the roles of lymphatic vessels in tumor growth and metastasis were neglected. However, it is well known that lymphatic metastasis is mainly responsible for the spread of epithelial malignant tumors, and its metastasis degree is closely related to the prognosis of patients. Consisting of simple endothelium, without tight junction, and incomplete, even defect of basic membrane, accordingly, the permeability of small lymphatic vessels is larger than that of small blood vessels, and it is beneficial to tumor metastasis. However, up to now, it is still controversial about intratumoral lymphangiogenesis, mechanism of lymphangiogenesis and relationship between lymphangiogenesis and tumor metastasis.

VEGF-C family is a group of highly conservative secreted glycoproteins, which regulate vasculogenesis, lymphangiogenesis, and vascular permeability, and have been implicated in many physiological and pathological processes[6-8]. VEGF-C is a member of the VEGF family, its encoding gene is located in 4q34, VEGF-C has 7 extrons and shares 30% autologousity with VEGF. The studies showed that VEGF-C expressed in normal placenta, ovary, small intestine, skeleton muscle, spleen, colon, *etc.* [9-12].

VEGFR-3/FLT-4, which belongs to the platelet-derived growth factor for receptor subfamily of receptor tyrosine kinase, was extensively expressed in embryonic tissues[13-16]. In adult however, VEGFR-3/FLT-4 was mainly expressed in lymphatic endothelia, so it was regarded as a special marker of lymphatic endothelia. The density and distribution of lymphatic vessels could be detected by VEGFR-3/FLT-4 antibody[17-21].

Recent studies showed that, the binding of VEGF-C to its special receptor FLT-4, could induce tyrosine phosphorylation of receptors, activate MAPK via intracellular kinase reaction, and finally promote proliferation of lymphatic endothelia, and lymphangiogenesis[22-25].

Karpanen' s results showed that VEGF-C could promote growth of tumor cells, which was correlated with the growth of lymphatic vessels around tumors and the intralymphatic spread of cancer[26]. Several clinical studies have proved that overexpression of VEGF-C in solide tumors, such as squamous cell carcinomas of head and neck, thyroid carcinoma, prostate cancer, gastric cancer, colorectal carcinoma, breast cancer, esophageal squamous carcinoma, cervical cancer, lung cancer, was relevant to lymphatic spread[27-31].

Akagi[32] examined the relationship between expression of VEGF-C in 99 primary tumors and 18 metastatic lymph nodes from colorectal cancer and clinicopathological features by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immumohistochemistry. The results showed that the expression of VEGF-C was correlated with lymphatic involvement, lymph node metastasis, and depth of invasion. To study the correlation between lymphogenous metastasis in early gastric carcinoma (EGC) and the expression of VEGF-C, Kabashima[33] selected 35 cases of lymph node metastasis-positive [n(+)] EGC and 70 cases of lymph node metastasis-negative [n(-)] EGC. Clinicopathologically, there were significant differences in median size, lymphatic invasion, and venous invasion between the two groups, the incidence of positive-expression of VEGF-C in n(+) EGC was significantly higher than that in n(-) EGC. In breast cancer, the expression of VEGF-C was correlated with lymphatic vessel invasion, the 5-year disease free survival rate of the VEGF-C positive group was significantly poorer than that of the VEGF-C negative group[34]. The expression of VEGF-C in breast cancer cells could increase intratumoral lymphangiogenesis, resulting in significantly enhanced metastasis to regional lymph nodes and lungs, and the degree of tumor lymphangiogenesis was highly correlated with the extent of lymph node and lung metastases[35].

Most of the studies demonstrated that VEGF-C was mainly produced by cancer cells, and VEGFR-3/FLT-4 by endothelial cells of the stromal lymphatic vessels, the incidence of VEGF-C was closely correlated with VEGFR-3/FLT-4 expression in the primary tumors. VEGF-C produced by cancer cells might induce the expression of VEGFR-3/FLT-4 on lymphatic endothelial cells, consequently, the activation of VEGF-C/FLT-4 paracrine loop induced the lymphangiogenesis[36].

However, the study by Gumingham[37] obtained opposite results, in which a significant correlation was found between expression of VEGF-C and VEGFR-3/FLT-4 in breast cancer, but no significant difference in VEGF-C expression between normal and neoplastic breast tissues and no association between VEGF-C and either lymph node status or number of involved nodes, patient age, tumor size, estrogen receptor status, or tumor grade were found.

We studied the correlation between VEGF-C and VEGFR-3/FLT-4 expression in 80 primary gastric cancers and adjacent normal tissues by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immumohistochemistry. The results showed that there was a significant difference in VEGF-C expression between primary tumor and adjacent normal tissue samples, and a significant correlation between expression of VEGF-C and VEGFR-3/FLT-4 in tumor tissues. The present study also clearly demonstrated that expression of VEGF-C and VEGFR-3/FLT-4 was closely related to the histological type of tumors and lymph node metastasis. Expressions of VEGF-C and VEGFR-3/FLT-4 were detected both in primary gastric cancer and in adjacent normal tissues. However, the former was significantly higher than the latter. Meanwhile, VEGFR-3/FLT-4 expression was detected in endothelial cells of stromal blood vessels and lymphatic vessels.

Tumor cell metastasis to regional lymph nodes is an early event in tumor spread, and this index is frequently used to predict disease prognosis[38]. VEGF-C is the first growth factor related to lymphangiogenesis, and the study of lymphangiogenesis of tumors is only at its beginning. Its mechanisms and mediators await further studies.

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• LIVER CANCER •

Activating mechanism of transcriptor NF-kappaB regulated by hepatitis B virus X protein in hepatocellular carcinoma

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Abstract

AIM: To investigate the mechanism and significance of NF-κB activation regulated by hepatitis B virus X protein (HBx) in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: The expression levels of HBx, p65, IκB-α and ubiquitin were detected by immunohistochemistry in HCC tissue microarrays (TMA) respectively, and IκB-α was detected by Western blot in HCC and corresponding liver tissues.

RESULTS: The percentage of informative TMA samples was 98.8% in 186 cases with a total of 367 samples. Compared with corresponding liver tissues (60.0%), the HBx expression was obviously decreased in HBV-associated HCC (47.9%, u=2.24, P<0.05). On the contrary, the expressions of p65 (20.6% *vs* 45.3%, u=4.85, P<0.01) and ubiquitin (8.9% *vs* 59.0%, u=9.68, P<0.01) were notably elevated in HCC. In addition, IκB-α had a tendency to go up. Importantly, positive relativity was observed between HBx and p65 (χ^2=10.26, P<0.01), p65 and IκB-α (χ^2=16.86, P<0.01), IκB-α and ubiquitin (χ^2=8.90, P<0.01) in HCC, respectively.

CONCLUSION: Both active and non-active forms of NF-κB are increased in HBV-associated HCC. Variant HBx is the major cause of the enhancement of NF-κB activity. The activation always proceeds in nucleus and the proteasome complexes play an important role in the activation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant tumor with a poor prognosis. Hepatitis B virus (HBV) has been shown to be linked epidemiologically to the HCC development and about eighty percent of the tumors in China are induced by HBV. As a unique non-structure protein, hepatitis B virus X protein (HBx) performs a variety of biological functions, such as gene transactivation[1], interaction with p53[2], interference with host DNA repair[3], repression of physiological proteolysis[4], modulation of cell proliferation and apoptosis[5,6], induction of malignant cell migration[7,8]. These functions may play an important role in the initiation and development of HCC associated with HBV infection. NF-κB, a crucial transcriptor, takes part in almost all aspects of cell regulation, including immune cell activation, stress response, proliferation, apoptosis, differentiation and oncogenic transformation. Currently, more attentions have been paid to the carcinogenesis of HBx transactiving NF-κB[9]. However, the active state of NF-κB in HCC has been seldom studied. As a new high-throughput technology introduced in 1999, tissue microarray (TMA) is worthy of popularization. In our study, the expression levels of HBx, p65, IκB-α and ubiquitin were detected by immunohistochemistry on TMA respectively, as well as IκB-α was detected by Western blot, in order to investigate the mechanism and significance of HBx activating NF-κB.

MATERIALS AND METHODS

Tissue samples

Paraffin specimens were prepared from operatively-resected HCC and non-HCC counterparts between 1997 and 2000, including 171 cases of serum HBV-positive HCC, 10 cases of serum HBV-negative HCC and their corresponding liver tissues, 5 cases of normal control liver tissues (Table 1). In addition, 24 couples of fresh HCC and its corresponding liver tissues were collected between March and October in 2001, stored at -80 ℃ until experiment.

Tissue microarray construction

All formalin-fixed and paraffin-embedded HCC tissues used in this study were sectioned and stained with hematoxylin-eosin (H&E). The H&E-stained sections were carefully diagnosed, and the representative regions of the tumor and its corresponding liver tissue for microarray were defined as well. HCC TMA was constructed according to the procedure described by Kononen *et al*[10]. Briefly, core tissue specimens, 0.6 mm in diameter, were taken from selected regions of individual donor blocks and precisely arrayed into recipient paraffin blocks (45 mm×22 mm) using a tissue-arraying instrument (Beecher Instruments, Silver Spring, MD, USA). After construction, the recipient paraffin block was incubated at 37 ℃ for one hour and the surface of the block was smoothed. Five-micrometer consecutive sections of this TMA block were cut with a microtome. The presence and morphology of tumor and liver tissues on arrayed samples were identified by H&E stained sections.

Immunohistochemistry

TMA section was deparaffinized through xylene and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 0.3% H_20_2 diluted in methanol for 30 min at room temperature. Antigen retrieval was performed by treating the slide in citrate buffer in a microwave for 10 min. The slide was incubated in a moist chamber with HBx mouse monoclonal

antibody (1:100; Chemicon, USA), p65 mouse monoclonal antibody (Santa Cruz, USA), IκB-α rabbit polyclonal antibody (Santa Cruz, USA) and ubiquitin rabbit polyclonal antibody (Neomarkers, USA) at 4 ℃ overnight respectively. After a brief wash in PBS, the slide was treated with goat anti-mouse antibody and goat anti-rabbit antibody (EnVision™ +Kits, DAKO, Denmark), respectively, for 45 min at 37 ℃. After a brief wash in PBS, the slide was developed in 0.05% freshly prepared diaminobenzedine solution (DAB, Sigma, St. Louis, MO) for 8 min, and then counterstained with hematoxylin. More than 5% cells stained were identified as a positive result.

Western blot

Western blot was carried out based on the protocol of *molecular clone*[11]. Briefly, frozen tissues were lysed in a single eradicator buffer (150 mmol/L NaCl, 50 mmol/L pH 8.0 Tris-HCl, 0.02% natriumazid, 1 ug/ml aprotinin, 100 ug/ml PMSF, 1% Triton X-100) and quantified by BCA method. The samples were boiled, loaded, separated on 12% SDS gel electrophoresis, transferred to nitrocellulose membrane, and reacted with IκB-α rabbit polyclonal antibody (1:1 000, Santa Cruz, USA). At last, the membrane was exposed several minutes after ECL substrate incubation.

Statistical analysis

HBx, p65, IκB-α, ubiquitin expression differences between HBV-associated HCC and corresponding liver tissues were analyzed statistically using *u* test. The relativity between HBx, IκB-α, p65, ubiquitin was analyzed statistically using χ^2 test or adjusted χ^2 test.

RESULTS

Tissue microarray

In this study, two HCC TMA blocks were constructed which contained a total of 181 cases with 367 samples. One thousand four hundred fifty-one informative samples were totally detected by immunohistochemistry on arrays and the observed ratio was up to 98.8% (1 451/1 468 samples). HE-stained sections showed that the morphology of tissues and cells could be seen clearly. The HCC array HE-stained sections and several types of the tumor are shown in Figure 1.

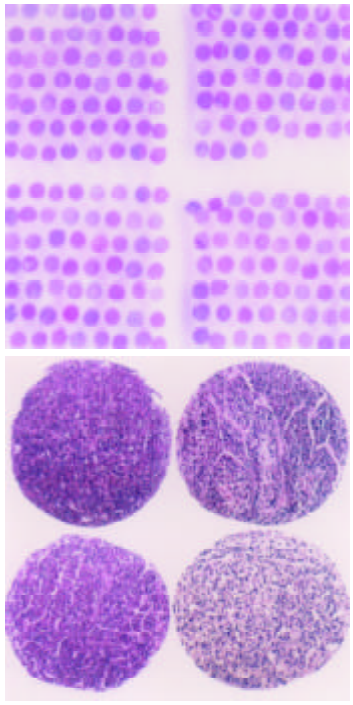

Figure 1 Overview of HCC TMA. A: TMA overview of H&E-staining section, B: HCC morphology on TMA stained by H&E.

Table 1 Clinicopathologic information of patients used in TMA

Group	HBV			Sex		Age	Diameter	Grade			Cirrhosis	
	Total	Positive	Negative	Man	Female	mean±SD (year)	mean±SD (cm)	II	III	IV	Yes	No
HCC	181	171	10	158	23	49.3±10.6	7.2±3.5	28	141	12	166	15
Normal	5	0	5	2	3	42.4±9.6	9.3±5.8				0	5

Table 2 Expressions of HBx, p65, IκB-α and ubiquitin in HCC and corresponding liver tissues

Group	HBx			p65			IκB-α			Ubiquitin		
	Total	Positive	%	Total	Positive	%	Total	Positive	%	Total	Positive	%
HCC	169	81	47.9	170	77	45.3	170	124	72.9	166	98	59.0
Control	170	102	60.0	170	35	20.6	171	116	67.0	168	15	8.9
Statistics		u=2.24[a]			u=4.85[b]			u=1.19			u=9.68[b]	

[a]P<0.05, [b]P<0.01.

Table 3 Relativity analysis between HBx, IκB-α, p65 and ubiquitin in HCC and corresponding liver tissues

		HBx		P65		Ubiquitin	
		Negative	Positive	Negative	Positive	Negative	Positive
HBx	Negative			58(52)	30(16)	39(57)	46(8)
	Positive			33(83)	47(19)	28(95)	52(7)
Statistics				χ^2=10.26[b](0.60)		χ^2=2.02(1.44)	
IκB-α	Negative	29(21)	17(34)	36(46)	9(9)	26(50)	17(4)
	Positive	59(47)	63(68)	56(89)	68(26)	42(103)	80(11)
Statistics		χ^2=2.28(0.11)		χ^2=16.86[b](1.02)		χ^2=8.90[b](0.04)	

[b]P<0.01, *vs* Corresponding liver tissue.

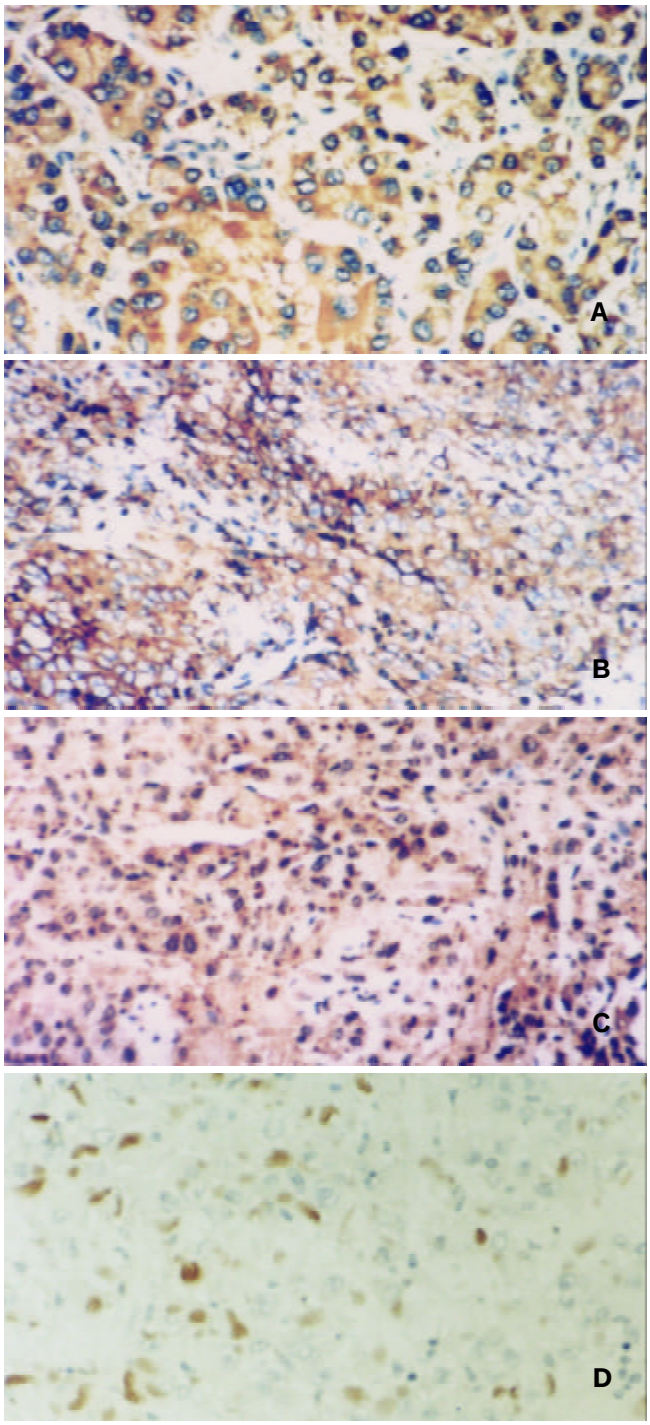

Figure 2 Expressions and locations of HBx, p65, IκB-α and ubiquitin in HCC detected by immunohistochemistry, EnVision ×200. A: HBx expression in cytoplasm, B: p65 immunostaining in cytoplasm and nuclei, C: IκB-α distribution in cytoplasm and nuclei, D: ubiquitin location in nuclei.

Expression differences of four proteins in HCC and corresponding liver tissues

The expression of HBx was restricted exclusively to cytoplasmic location in both HCC and liver tissues. The positive immunostainings of p65 and IκB-α were seen only in cytoplasm of liver tissues, but in both cytoplasm and nuclei of HCC. The positive signal of ubiquitin was distributed predominantly in cytoplasm of liver tissues and in nuclei of HCC (Figure 2).

In serum HBV-positive cases, the HBx expressions in HCC (81/169, 47.9%) were significantly decreased as compared with the corresponding liver tissues (102/170, 60.0%, $P<0.05$). On the contrary, the expressions of p65 and ubiquitin were notably elevated in HCC (45.3%, 59.0% respectively) as compared with corresponding liver tissues (20.6%, 8.9% respectively, $P<0.01$). The positive rate of immunostaining reaction of IκB-α in HCC and corresponding liver tissues was 72.9% and 67% respectively. The difference was not significant, though the staining in HCC was more intense (Table 2).

In serum HBV-negative cases, the expressions of HBx, p65, IκB-α, ubiquitin in HCC were detected in 2/10 cases, 5/9 cases, 7/9 cases and 5/10 cases respectively. In corresponding liver tissues, their expressions were detected in 2/10 cases, 2/9 cases, 7/9 cases and 2/10 cases respectively.

In five normal liver tissues, all expressions of HBx, p65 and ubiquitin were negative, whereas, IκB-α was demonstrated to be weakly positive.

Western blot of IκB-α

Compared with corresponding liver tissues, elevated levels of IκB-α were detected in 10 HCC cases, decreased in 1 case. In the other 13 couples of HCC and corresponding liver tissues, no obvious difference of expression was detected (Figure 3).

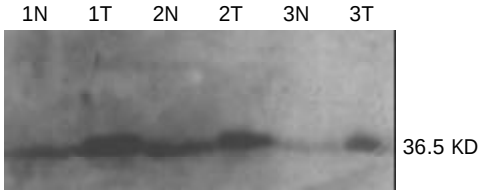

Figure 3 IκB-α expression levels detected by Western blot. IκB-α levels were elevated in 3 cases of HCC compared with their corresponding liver tissues.

Relativity analysis between HBx, IκB-α, p65 and ubiquitin

χ^2 test showed that no relativity existed between HBx, IκB-α, p65 and ubiquitin in corresponding liver tissues, but a positive relativity was observed between HBx and p65, p65 and IκB-α, IκB-α and ubiquitin in HCC (Table 3).

DISCUSSION

TMA is a new technology first introduced in 1999. It contains hundreds or even thousands of small tissue samples arranged into a grid for rapid, cost efficient and high-throughput analysis. TMA has been used widely in gene or protein expression analysis, antibody screening, tissue specificity detection of proteins, phenotype versus genotype analysis, RNA or DNA *in situ*-hybridization[10,12,13]. Based on TMA, we studied the expressions of HBx, NF-κB, IκB-α, ubiquitin and their interrelationship in HCC.

The result of HBx expression detection in HBV-associated HCC showed that HBx expression was reduced in cancer tissue as compared with the corresponding liver tissue. The result might be ascribed to the existing form of HBV in tumor tissue. Many researches indicated that integration was the major form of HBV in HCC, so the viral copies were far less than that in non-tumor tissues where free virus prevails[14]. The expression of HBx in samples with serum HBV-negative implicated quite a few patients were once infected with HBV. Moreover, the cytoplasmic location of HBx was consistent with the previous reports[15].

NF-κB plays a vital role in almost all aspects of cell regulation such as immune cell activation, proliferation, apoptosis, stress response, differentiation and oncogenic transformation. Activated NF-κB can mediate the expression of a large (more than 150) and diverse set of inflammatory and immune response mediators. It has been considered as a central regulator of cellular responses and played a pivotal

role both at the stage of initiation and perpetuation of chronic inflammation[16-19]. NF-κB is sequestered in the cytosol of unstimulated cells via non-covalent interactions with a class of inhibitor proteins, called IκBs. Signals that induce NF-κB activity cause the phosphorylation of IκBs, their dissociation and subsequent ubiquitination and degradation in 26S proteasome complex, allowing NF-κB proteins to enter the nucleus and induce gene expression by binding κB site in DNA. As one of the NF-κB activation products, IκB-α could induce NF-κB export from the nucleus by reuniting NF-κB and terminating associated gene activation. The negative feedback loop could keep NF-κB sensitivity to signals by returning rapidly to baseline activity[20-22].

Because p65 is one of the most common members in NF-κB family, and IκB-α is the most important inhibitor, detection of p65 and IκB-α expression can reflect the state of NF-κB metabolism. In our test, low expression rate of p65 and weakly positive expression of IκB-α represented the nonactive state of NF-κB in non-cancerous tissues, but the activation enhancement of NF-κB in HCC was in accord with the previous reports[23,24]. The expression of IκB-α in HCC and its corresponding liver tissue has not been reported yet. In our study, no statistical difference was found in positive rates between HCC and its corresponding liver tissue, the discrimination of immunostaining intensity was scented. Furthermore, Western blot validated the concentration augmentation of IκB-α in HCC, while in the meantime, molecular weight alteration was not observed. The results illustrated that IκB-α regulation gave priority to quantity change in HCC. The location of p65 and IκB-α represented the activation or non-activation form of NF-κB. Quite a few cytoplasm locations of p65 and IκB-α observed in our test accounted for the non-activated NF-κB increase in HCC, which contradicted with cytoplasm decrease of p65 and IκB-α observed *in vivo* system[25]. We ascribed the contradiction of NF-κB metabolism to the persistence in HCC and the transience *in vivo* system. The lasting activation of NF-κB can automatically regulate the production of IκB-α and possibly even p65 or p50. A great deal of non-activated NF-κB repertory is in favor of lasting activation of NF-κB.

IκB-α proteolysis is by the ubiquitin-proteasome pathway, but the exact locality is still not clear. Birbach *et al* expatiated IκB-α shuttle mechanism between cytoplasm and nucleus[26]. It is recognized now that besides IκB-α, up-stream kinases such as NIK and MAPKK can shuttle between cytoplasm and nucleus. The present study is the first to show the distribution and expression of ubiquitin in HCC. The results of nuclear location of ubiquitin and its relativity with IκB-α indicated the proteolysis of IκB-α was processed in tumor cell nuclei. Of course, another likelihood was that transcription of ubiquitin would be promoted after NF-κB activation[27]. There is no relativity between ubiquitin and HBx, so whether the proteolysis of HBx passes through non-ubiquitin pathway or not remains to be determined.

Positive relativity between HBx and p65 in HCC indicated that HBx existed in tumor tissues was one cause of inducing NF-κB activation. It has been found in some studies that HBx mutation was common in HCC compared with corresponding liver tissues[28]. Frequent types of HBx mutation were COOH-terminal truncation and hotspot mutations in certain amino acids[29-31]. COOH-terminally truncated HBx is encoded by truncated X gene, which usually derives from HBV integrated into host genome. Base mutation and frame-shift are the other causes. Usually, it was considered that HBx activated NF-κB relied on its transactivation domains, and COOH-terminal transactivation domain was important to its transactivation function[32,33]. In our study, NF-κB activation was elevated in HCC with a low HBx expression compared with corresponding liver tissues. Therefore, the results implied that HBx might activate NF-κB by other unknown mechanisms but not its transactivation ability. One likeness was that C-terminally truncated HBx lost its ability to suppress proteasome complex and facilitated IκB-α degradation and NF-κB activation[4]. The high percentage of ubiquitin expressions in HCC (59.0%) from our results in part supports the opinion. The detailed mechanism of NF-κB activation in HBV-associated hepatocellular carcinoma remains to be further studied.

In conclusion, NF-κB activation induced by HBx could not only facilitate infected cell survival and HBV escape from immune clearance, but also promote liver cell malignant transformation and tumor cell advantageous growth[34]. Variant HBx plays an important role in potentiating NF-κB activation.

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• LIVER CANCER •

Purification of heat shock protein 70-associated tumor peptides and its antitumor immunity on hepatoma in mice

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Abstract

AIM: To purify the heat shock protein (HSP) 70-associated tumor peptides and to observe its non-MHC-I molecule restrictive antitumor effect.

METHODS: By ConA-sepharose affinity chromatography, ADP-agarose affinity chromatography, and DEAE anion exchange chromatography, we were able to purify HSP70-associated peptides from mouse hepatoma (HCaF) cells treated in heat shock at 42 ℃. Specific active immunization and adoptive cellular immunization assay were adopted to observe the immunoprotective effect elicited by HSP70-associated peptide complexes isolated from HcaF.

RESULTS: The finally purified HSP-associated peptides had a very high purity and specificity found by SDS-PAGE and Western blot. Mice immunized with HSP70-associated peptide complexes purified from HCaF cells were protected from HCaF living cell challenge. This effect was dose dependent. Adoptive immunization of immune spleen cells of mice immunized with HSP70-associated peptide complexes could elicit immunity against HCaF challenge, and the tumor-free mice could resist repeated challenges. This effect could be continuously enhanced by repeated challenge with HCaF living cells. The tumor-free mice could tolerate the challenge for as high as 1×10^7 HCaF cells. The mice immunized once with spleen cells pulsed with HSP70-associated peptide complexes *in vitro* could also result in a certain adoptive immunity against HCaF.

CONCLUSION: High purity and specificity of HSP70-associated peptides could be achieved from tumor cells by the low-pressure affinity chromatography method used in this study. HSP70-associated peptide complexes derived from the HCaF can elicit non-MHC-I molecule restrictive immunoprotective effect against HCaF. This effect can be transferred by adoptive immunization to mice and enhanced by repeated challenge with HCaF live cells.

Chen DX, Su YR, Shao GZ, Qian ZC. Purification of heat shock protein 70-associated tumor peptides and its antitumor immunity on hepatoma in mice. *World J Gastroenterol* 2004; 10(3):361-365
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INTRODUCTION

Heat shock proteins (HSPs) are molecular chaperones which are emerging as biochemical regulators of cell growth, apoptosis, protein homeostasis and cellular targets of peptides. Numerous studies have demonstrated that HSP70 preparations derived from a tumor can elicit cancer-specific immunity against the same tumor by virtue of their ability to bind tumor-specific peptides[1-7]. Further studies indicated that tumor immunity elicited by immunization with HSP peptide complexes, including HSP70 and gp96 family, is mediated by $CD8^+$ T lymphocytes, and its mechanism involves MHC-I class molecule restricted response which is required to channel the peptides into class I presentation pathway[8-10]. In this study, we isolated successfully HSP70-associated peptides from mouse hepatoma HCaF by low-pressure chromatography system and investigated the non-MHC-I class molecule restrictive anti-tumor immunity elicited by purified HSP70 associated peptide complexes.

MATERIALS AND METHODS

Materials

Animals and tumor strain BALB/c mice ($H\text{-}2^d$), weighing 18-20 g, were purchased from Xipuerbikai Experimental Animal Ltd, Shanghai, China. Mouse hepatoma HCaF(non-MHC-I class molecule expression) was obtained from Cancer Institute, Dalian Medical University, China.

Reagents ConA-sepharose was purchased from Pharmacia Inc. ADP, ADP-agarose from Sigma Corp. Macro-Pre DEAE support, Macro-Prep High Q from Bio-Rad Corp. Low-molecule weight standard protein and IgG of goat anti-mouse labeled with horseradish peroxidase from B.M Corp, RPMI1640 and new born bovine serum from GIBCO Corp, and anti-HSP70 McAb(mouse anti-mouse) from Wuhan Boster Corp. All other reagents used were of analytic grade.

Methods

Purification of HSP70-associated peptides The ascites of mice which had been inoculated intraperitoneally with HCaF cells for 6-7 days were used. HCaF cells were washed three times in PBS, and then suspended in RPMI1640 complete medium with water immersion at 42 ℃ for 12 hours. The HCaF cell pellets harvested were homogenized in hypotonic buffer (10 mM $NaHCO_3$, 0.5 mM PMSF, pH7.1) and centrifuged at 100 000×*g* for 90 min at 4 ℃, and the supernatant was collected. The supernatant concentrated by PEG(MW600) was applied to a ConA-sepharose column in the presence of ConA-sepharose bound buffer C (20 mM Tris-acetate, pH 7.5, 0.5 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 15 mM 2 ME, 0.5 mM PMSF), and fluid was collected at a flow rate of 12 ml /h, that was the

ConA-sepharose unbound protein. The fraction was dialyzed against buffer D (20 mM Tris-acetate, pH7.5, 20 mM NaCl, 3 mM $MgCl_2$, 15 mM 2ME, 0.5 mM PMSF) overnight at 4 ℃. The sample was applied to an ADP-agarose column equilibrated previously with buffer D at a flow rate of 12 ml/h. The proteins were eluted by buffer D containing 0.5M NaCl and buffer D until the protein was not detected by Bradford method. The column was eluted by 25 ml buffer D containing 3 mM ADP. The harvested elute was concentrated and dialyzed against DEAE ion-exchange buffer A (20 mM Na_3PO_4, 20 mM NaCl, pH7.2). The sample was applied on a DEAE column equilibrated with buffer A at a flow rate of 10 ml/h. After buffer A equilibrium for 30 min, the target protein was eluted at a linear gradient of 20 mM-1 000 mM NaCl in buffer A (20 mM Na_3PO_4, 1M NaCl, pH7.0, ranging from 0%-100%). Various fractions harvested were detected with SDS-PAGE and silver staining. The fractions of HSP70 protein were collected, pooled and dried with freeze-drying, and stored at -20 ℃ until further use.

Identification of HSP70-associated tumor peptides HSP70 proteins were resolved on 10% SDS-PAG, subjected to electrophoresis, detected by silver staining, and blotted using mAb specific for HSP70. Manipulation of SDS-PAGE and Western blot were performed according to the method described by Sambrook *et al*[11]. The protein content was determined by Bradford standard curve method[12].

Active immunization assays BALB/c mice were immunized subcutaneouly with HSP70-associated peptide complexes, supernatant from homogenate of HCaF cells treated with heat shock (S-HCaF), supernatant from homogenate of liver cells treated with heat shock (S-HC), and PBS twice at weekly intervals separately and challenged by subcutaneous injection of the indicated number of HCaF living cells (5×10^4 cells in 100 μl PBS) one week after the last immunization.

Adoptive immunoprotection experiment BALB/c mice were immunized with tail vein injection of immune spleen cells (ISC, 1×10^7 cells in 200 μl PBS) of mice immunized with HSP70-associated peptides and free of tumor, twice at 5 days intervals, and challenged subcutaneouly by 5×10^4 HCaF living cells in 100 μl PBS 3 days after the last immunization. The mice with complete protection were challenged by 1×10^5 HCaF living cells again 50 days after the first challenge. The mice which tolerated the second HCaF challenge, were challenged by 1×10^7 HCaF living cells again. In another experiment, the mice were immunized by tail vein injection of spleen cells (1×10^7 cells in 200 μl PBS) pulsed with HSP70-associated peptides *in vitro* and challenged subcutaneouly by 5×10^4 HCaF living cells in 100 μl PBS 3 days after immunization. Corresponding control groups were set in above experiment.

Statistical analysis

Values were expressed as mean±SD or percent (%). The data were analyzed with SPSS 8.0 software package. The results were considered statistically significant when $P<0.05$.

RESULTS

Purity and specificity of HSP70-associated peptides

Purified HSP70-associated peptides showed one was bond on SDS-PAGE (Figure 1). Western blotting showed that molecular weight of HSP70-associated peptides purified from HCaF was about 70KD, which was consistent with the expected maker (Figure 2). The results indicated that HSP70-associated peptide complexes isolated from HCaF had a very high purity and specificity.

Active immunoprotective effect of HSP70-associated peptides derived from HCaF

As shown in Figure 3 and Table 1, different degrees of immunoprotection against HCaF challenge could be elicited by immunization with HCaF-70 associated peptide complexes derived from HCaF. The effect on female groups was much better than that on male groups. This effect was dose-dependent. In the female groups, mice immunized with 600 μg/kg HSP70-associated peptides showed better protection than those immunized with 300 μg/kg HSP70-associated peptides. The survival rate of the two groups was 83.3% and 60%, respectively. The female mice immunized with S-HCaF also could result in a certain extent of protective effect against HCaF challenge, and the survival rate and mean survival time of tumor-bearing mice had significant differences from S-HC and PBS control groups ($P<0.01$).

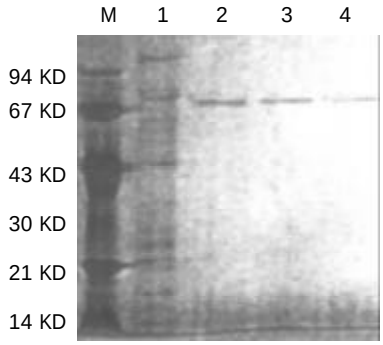

Figure 1 SDS-PAGE analysis of HSP70-tumor peptide complexes (silver staining). M: protein molecular weight marker, 1: protein eluted with 250-350 mmol/L NaCl buffer, after ADP-agarose chromatography (eluted with 0.5 mol/L NaCl buffer, PH7.5, containing 20 mmol/L Tris-acetate) and DEAE-ion exchange, 2-4: protein eluted with 250-350 mmol/L NaCl buffer, after ADP-agarose chromatography (eluted with 3 mmol ADP/L buffer, pH7.5, containing 20 mmol/L Tris-acetate) and DEAE-ion exchange.

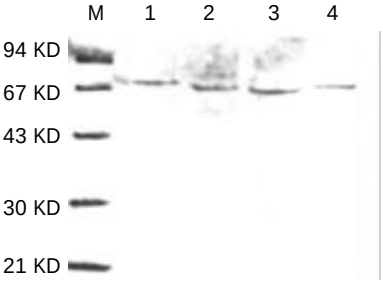

Figure 2 Western blot identification of HSP70-tumor peptide complexes purified from HCaF cells. The notes are the same with Figure 1.

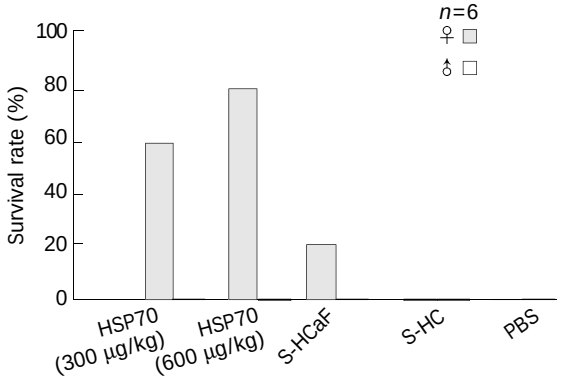

Figure 3 Immunoprotective effect of HSP70-associated peptides against tumor.

Table 1 Comparison of survival time of tumor-bearing mice after immunized by HSP70-peptide complexes

Groups	Sex	No. of tumor-bearing / No. of mice challenged	Survival days of tumor-bearing (mean±SD)	Extended survival rate (%)
HSP70 300 μg/kg	Female	2/6	48.0±8.5[ad]	67.4[bd]
	Male	6/6	31.0±13.2	49.7[c]
600 μg/kg	Female	1/6	33	
	Male	6/6	30.7±12.7	48.4[c]
S-HCaF	Female	5/6	59.2±10.0[bd]	106.5[bd]
	Male	6/6	20.3±0.9	-2.0
S-HC	Female	6/6	24.0±5.8	-16.3
	Male	6/6	18.5±0.6	-10.5
PBS	Female	6/6	28.7±11.6	
	Male	6/6	20.7±6.1	

[a]$P<0.05$, *vs* PBS; [b]$P<0.01$, *vs* PBS; [c]$P<0.01$, *vs* S-HC; [d]$P<0.05$, *vs* S-HC.

$$\text{Extended survival rate} = \frac{\text{MST of experimental group - MST of control group}}{\text{MST of control group}} \times 100\%$$

Table 2 Comparison of tumor growth in mice immunized with HSP70-peptide complexes

Groups	Sex	No. of tumor-bearing mice	10 days after HCaf challenge		15 days after HCaF challenge	
			Size of tumor (mm^3)	Inhibition rate (%)	Size of tumor (mm^3)	Inhibition rate (%)
HSP70 300 μg/kg	Female	2	0.88[bc]	97.0[ac]	6.66[bd]	98.5[ad]
	Male	6	61.98[bd]	80.2[bd]	384.77[bc]	83.2[bc]
600 μg/kg	Female	1	0.45	98.4	5.74	
	Male	6	64.49[b]	79.4[bd]	515.10[bc]	77.6[bc]
S-HCaF	Female	5	3.09[bc]	89.2[bc]	10.21[bc]	97.7[bc]
	Male	6	24.27[bd]	92.2[bd]	501.55[b]	78.2[bd]
S-HC	Female	6	30.00	-4.5	402.17	8.2
	Male	6	204.89	34.5	1 554.30	32.3
PBS	Female	6	28.70		438.17	
	Male	6	312.83		2 295.52	

[a]$P<0.05$, *vs* PBS; [b]$P<0.01$, *vs* PBS; [c]$P<0.01$, *vs* S-HC; [d]$P<0.05$, *vs* S-HC.

$$\text{Inhibition rate (\%)} = 1 - \frac{\text{Tumor size of experimental group}}{\text{Tumor size of control group(PBS)}} \times 100\%$$

Table 3 Comparison of tumor weight and spleen weight after tumor-bearing mice death (mean±SD)

Groups	Female			Male		
	No. of tumor-bearing mice	Tumor wt. (g)	Spleen wt. (g)	No. of tumor-bearing mice	Tumor wt. (g)	Spleen wt. (g)
HSP70 300 μg/kg	2	14.40±6.22[bce]	0.93±0.18[bde]	6	7.06±2.59	0.19±0.04
600 μg/kg	1	6.5	0.4	6	7.51±2.33	0.20±0.11
S-HCaF	6	8.68±1.65[a]	0.53±0.22[be]	6	5.06±0.71	0.24±0.10
S-HC	5	4.72±1.65	0.17±0.08	6	4.98±1.04	0.14±0.02
PBS	6	6.78±1.34	0.25±0.06	6	6.30±2.18	0.12±0.07

[a]$P<0.05$, *vs* S-HC; [b]$P<0.01$, *vs* S-HC; [c]$P<0.05$, *vs* S-HCaF; [d]$P<0.01$, *vs* S-HCaF; [e]$P<0.01$, *vs* PBS.

Table 4 Adoptive immunoprotective effect against HCaF challenge elicited by ISC transfer

Group	Survival rate (%) (No. of death / No. of mice challenged)	Survival days of tumor-bearing mice (mean±SD)	Extended survival rate (%)	Tumor wt. (g) (mean±SD)	Spleen wt.(g) (mean±SD)
ISC	75 (2/8)[a]	50.5±9.2[b]	71.0[b]	14.92±0.42[bc]	0.39±0.01[bc]
SC	0 (8/8)	36.8±12.9	24.6	7.73±2.14	0.18±0.05
Chal. control	0 (8/8)	29.5±3.9	0	7.35±1.16	0.21±0.05

[a]$P<0.05$, *vs* chal. Control; [b]$P<0.01$, *vs* chal. Control; [c]$P<0.01$, *vs* SC.

On the 10th day and 15th day after HCaF challenge, the tumor volume of tumor-bearing mice in both HSP70-associated peptides and S-HCaF groups was apparently smaller than that in S-HC and PBS groups (Table 2). In females, tumor weight and spleen weight of dead mice in both HSP70-associated peptides and S-HCaF groups were significantly larger than those in S-HC and PBS groups (Table 3).

Adoptive immunprotective effect

Adoptively transferred immune spleen cells of mice, which had been immunized with HSP70-associated peptides and were free of tumor, could provoke immunoprotection against HCaF challenge. The survival rate of ISC-immunized mice was 75%, and the mean survival time of tumor-bearing mice was significantly prolonged compared with both non-immune spleen cells (SC) group and challenge control (Table 4).

The mice which tolerated HCaF challenge were challenged by 1×10^5 HCaF living cells, and the survival rate was 83.3%, while all of the challenge controls died within 27.5 days. The mice which tolerated the second challenge could tolerate repeated challenges for as high as 1×10^7 HCaF living cells, while all the challenge controls died within 22 days (Figure 4).

The mice immunized once with spleen cells pulsed with HSP70-associated peptides *in vitro* could lead to a certain extent of protection against HCaF challenge. Although the survival rate of mice was only 20%, the mean survival time of tumor-bearing mice was 42 days, all the mice treated with S-HCaF, S-HC, SC or PBS died within 33 days.

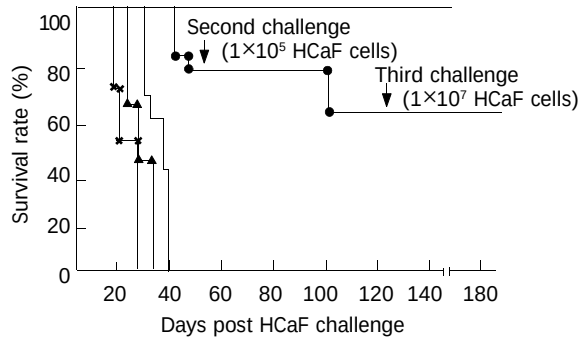

Figure 4 Protective effect of transferred immune spleen cells against repeated challenges with HCaF. – ▲-▲ ×-× First, second, and third challenge controls. ●-● Transferred immune spleen cell group.

DISCUSSION

Intracellular HSP70 is very low in content of cells and makes up approximately 0.01% of the cell wet weight. At present, ConA-sepharose affinity chromatography or ADP-agarose affinity chromatography in combination with fast protein liquid chromatography[13,14] has been the typical method for purifying HSP70. The recovery of HSP70 isolated by the above methods was lower than 50 mg /L cell pellet[4]. The purification protocol used in our experiment resulted in a relatively high recovery compared with the traditional method of HSP70 purification, being for 50-100 mg /L wet weight of cell pellet. This purification method might be used as a universal technique due to its easy and reproducible isolation of antigenic HSP from other tissues of different sources.

Numerous investigations have shown that HSP itself had no antigenicity and its immunogenicity has been attributed to the peptide chaperoned carried by itself[1-4]. In this experiment, tumor rejection assay demonstrated that HSP70 purified from HCaF could elicit tumor immunity. We therefore conclude that purified HSP70 identified by both SDS-PAGE and Western blot should be regarded as HSP70-associated tumor peptides.

Our experiment indicated that HSP70-associated peptides derived from HCaF could elicit anti-tumor immunity. Mice immunized with 600 μg/kg HSP70-associated peptides showed better protection than those immunized with 300 μg/kg. This effect was dose-dependent, and was consistent with other reports[4]. In this study, we found the transferred immune spleen cells of mice immunized with HSP70-associated peptide complexes could elicit immunity against HCaF challenge, and the tumor-free mice could resist repeated HCaF challenges. This effect could be continuously enhanced by repeated challenges with HCaF living cells. The mice so treated could tolerate a challenge for as high as 1×10^7 HCaF cells. Our results demonstrated that adoptively transferred immune spleen cells immunized with HSP70-associated tumor peptides could result in immunoprotection against the same tumor. This evidence indicates that anti-tumor immunity elicited by HSP70-associated peptides has a considerable stability of immunoprotection and specific immunologic memory.

It has been generally believed that tumor immunity elicited by immunization with exogenous HSP70-peptide complexes is mediated by antigen-presenting cells and presented by MHC class I molecules[15-24]. It is worth pointing out that the tumor model of HCaF used in our experiment did not express MHC class I molecule protein. Therefore, the tumor immunity elicited by HSP70-associated peptides derived from HCaF might not be mediated by $CD8^+$ CTL. Several studies have shown that HSP70-associated peptides could directly activate $\gamma\delta^+$T lymphocytes or nature killer cells as superantigen without being dependent on the stimulation of MHC-Ia and I b class molecules[25-30]. It is possible that HSP70-associated peptides derived from HCaF can elicit antitumor immunity in a similar manner.

In addition, we found that the supernatants of HCaF cell homogenate could also result in a certain tumor immunity. This effect might be related to the expression of HSP in HCaF cells induced by heat shock.

Compared spleen weight of tumor-bearing mice in various groups, the mean spleen weight of tumor-bearing mice in HSP70-associated peptide complexes group was significantly higher than that in the controls. Our results further showed that adoptively transferred spleen cells pulsed with purified HSP70-associated peptides could also provoke a certain protection against HCaF challenge. These results indicated that spleen cells might play an important role in tumor immunity mediated by HSP70-associated peptides.

It is of great interest to note that the protective effect in the female mice immunized with HSP70-associated peptide complexes was significantly better than that in the male group. The difference may be associated with estrogens, its mechanisms remain to be explored further.

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• LIVER CANCER •

Antisense oligonucleotide targeting at the initiator of hTERT arrests growth of hepatoma cells

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Abstract

AIM: To evaluate the inhibitory effect of antisense phosphorothioate oligonucleotide (asON) complementary to the initiator of human telomerase catalytic subunit (hTERT) on the growth of hepatoma cells.

METHODS: The as-hTERT was synthesized by using a DNA synthesizer. HepG2.2.15 cells were treated with as-hTERT at the concentration of 10 μmol/L. After 72 h, these cells were obtained for detecting growth inhibition, telomerase activity using the methods of MTT, TRAP-PCR-ELISA, respectively. BALB/c(nu/nu) mice were injected HepG2.2.15 cells and a human-nude mice model was obtained. There were three groups for anti-tumor activity study. Once tumors were established, these animals in the first group were administered as-hTERT and saline. Apoptosis of tumor cells was detected by FCM. In the 2nd group, the animals were injected HepG2.2.15 cells together with as-hTERT. In the third group, the animals were given as-hTERT 24 hours postinjection of HepG2.2.15 cells. The anti-HBV effects were assayed with ELISA *in vitro* and *in vivo*.

RESULTS: Growth inhibition was observed in cells treated with as-hTERT *in vitro*. A significant different in the value of A_{570}-A_{630} was found between cells treated with as-hTERT and control ($P<0.01$) by MTT method. The telomerase activity of tumor cells treated with as-hTERT was reduced, the value of A_{450} nm was 0.42 compared to control (1.49) with TRAP-PCR-ELISA. The peak of apoptosis in tumor cells given as-hTERT was 21.12%, but not seen in saline-treated control. A prolonged period of carcinogenesis was observed in the second and third group animals. There was inhibitory effect on the expression of HBsAg and HBeAg *in vivo* and *in vitro*.

CONCLUSION: As-hTERT has an anti-tumor activity, which may be useful for gene therapy of tumors.

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is a critical disease threatening human health. It is one of the most common malignant tumors worldwide, however there is no effective treatment at present. It is very necessary to explore new methods for the therapy of HCC. The developments in molecular biochemistry have afforded the possibility for this purpose.

Telomerase, a ribonucleoprotein enzyme that synthesizes telomeric DNA, is thought to be necessary for cellular immortality and carcinogenesis[1,2], and consists of human telomerase RNA component (hTERC), human telomerase protein 1(hTEP1), and human telomerase reverse transcriptase (hTERT). Among them, only the expression of hTERT mRNA is correlated with telomerase[3], and mainly regulates the expression of human telomerase enzymatic activity[4-6]. hTERT is a useful marker for telomerase activation[7,8]. It is associated with the majority of malignant human cancers. Therefore, telomerase is the target of anti-tumor drugs research. For example, many scientists declared that ribozyme which cleavaged telomerase mRNA and PS-ODNs complementary to the repeat sequence of the mammalian telomere (5' TTAGGG3') could arrest tumor cells [9-13] in cell lines. In this study, we observed the inhibitory effect of as-hTERT both *in vivo* and *in vitro*.

In HCC, the activity of telomerase played a very important role during the occurrence of HCC and cirrhotic livers[14]. The positive hTERT mRNA was 89.47% in HCC tissues[15]. Though many factors were related to hepatocarcinogenesis[16], infection of HBV and HCV[17] was the most important. Tahara *et al*[18], discovered that telomerase activity was 100% in HCC tissues with HBV positivity. These studies indicated that there might be a close relationship between HBV infection and telomerase activity during hepatocarcinogenesis. Therefore, in this study, HepG2.2.15 cells, a cell line in which HBV genome was integrated into the chromosome, were the target cells and as-hTERT complementary to hTERT promoter was added to these cells, and the anti-tumor effects and inhibitory action on HBV gene expression of as-hTERT were studied *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

The oligomers used in this study were prepared by Shenggong Co. The solutions were suspended in sterile phosphate-buffered saline(PBS) and filtered into 1.5 ml sterile Eppendorf tube, and stored at 4 ℃ until use. AsON used in this study targeted at the promoter area of human hTERT with the sequence 5' GCC ACG TGG GAA GCG 3' (-192--176 site). In this area, there is a potential binding site of upstream stimulating factors, such as pro-oncogene c-myc. The random sequence (5' TTG CCG AGC GGG GTA 3') was used as control. HepG2.2.15 was used as targeted cells. This cell line was purchased from Beijing Institute of Medical Biology. Fetal calf serum (FCS) and G418 were purchased from GIBCO and Sigma respectively. The quantitative detection kit of telomerase (PCR-ELISA) was purchased from Roche Molecular Biochemicals, Germany.

Cell culture

The experimental protocols were similar to those as previously described[19,20]. HepG2.2.15 was a hepatoma cell line which was integrated with HBV genome, and could replicate intact HBV DNA and extract HBsAg and HBeAg. The cells were grown *in vitro* in a 25 ml flask at a concentration of 5×10^4 cells per milliliter in MEM media supplemented with 10% fetal bovine serum containing 380 μg/ml G418[21,22]. The cells were tested for HBsAg and HBeAg and found to replicate HBV DNA. When the cells grew to 75% confluence, they were digested and used for the *in vitro* and *in vivo* studies.

MTT assay

The cells were cultured in 60 mm^2 dishes with 2×10^5 cells per dish. The next day as-hTERT and control sequence were added at a concentration of 10 μM. After 72 h incubation, these cells were added 20 μL of MTT per dish. Four hours later, the cells were given 100 μL of DMSO per dish and the absorbance of the sample was measured at 570 nm and 630 nm using an ELISA microtiter plate reader. The value of absorbance at 570 nm and 630 nm was named A_{570} and A_{630}.

Determination of telomerase activity

Telomerase activity assay was performed according to a polymerase chain reaction-based telomeric repeat amplification protocol as described previously[23-26] using a kit from Roche (Germany). The cells (10^5-10^6 per dish) were harvested after 72 h incubation with as-hTERT and control sequence.

Preparation of extracts from cells The cells were put in a 1.5 ml Eppendorf tube and pelleted at 3 000×g for 10 min in a refrigerated centrifuge at 4 ℃. Then these cells were washed once in cold PBS. For one tube, 200 μl pre-cooled lysis reagent was added and incubated on ice for 30 min and centrifuged at 10 000×g for 20 min at 4 ℃. A total of 175 μl supernatant was carefully transferred to a fresh Eppendorf tube. These cell extracts were used for the tolemerase assay using the telomerase PCR-ELISA procedure.

Telomeric repeat amplification protocol (TRAP reaction) For each sample, 25 μl reaction mixture was transferred into a PCR tube. Three μl cell extract and sterile water were transferred to a final volume of 50 μl. Then a combined primer elongation reaction was performed according to the following protocol (Table 1).

Table 1 Protocol of TRAP reaction

Step	Time	Temperature	Cycle number
Primer elongation	30 min	25 ℃	1
Telomerase inactivation	5 min	94 ℃	2
Amplification			3-32
Denaturation	30 s	94 ℃	
Annealing	30 s	50 ℃	
Polymerization	90 s	72 ℃	
	10 min	72 ℃	33

Hybridization and ELISA procedure The product of PCR was aliquoted, denatured, bound to a streptavidin-coated 96 well plate, and hybridized to a DIG-labelled telomeric-repeat specific probe. An antibody to DIG conjugated to peroxidase was subsequently bound to DIG. Then tetramethyl benzidine (TMB) was metabolized, the substrates of the enzyme was added to produce a colored reaction product. The absorbance of the sample was measured at 450 nm using an ELISA microtiter plate reader within 30 min after adding the stop reagent. The value of absorbance at 450 nm was named A_{450}.

Animal studies

BALB/c (*nu/nu*) mice (weighing approximately 18-20 g) were purchased from the Experimental Animals Center of Chinese Academy of Sciences. The mice were divided into eight teams randomly. These animals were placed into fresh cages with free access to sterile water. All the solutions were filtered into sterile bottles. In order to compare the different curative effects of as-hTERT by different administration routes, the study was divided into three study groups.

Group I: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla and tumor progression was measured daily. Two weeks postinjection, the mice with tumors about 0.5 cm in diameter were selected, and as-hTERT and control sequencewere injected into the tumors subcutaneously. These animals were divided into two teams, one team (n=12) was injected as-hTERT 100 μg/mouse/day, the other (n=6) was treated with saline 100 μl/mouse/day. Five days postinjection, the mice were killed by removing the eyeballs and the tumor tissues were removed, and stored in 10% formalin for FCM. These tumor samples were also embedded in paraffin, and sectioned into slices about 5 μm in thickness. The sections were stained with hematoxylin and eosin for histological examination. In this group, the serum were harvested and stored at -20 ℃ for detecting HBsAg and HBeAg.

Group II: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla and as-hTERT 100 μg/mouse (n=7) simultaneously, the controls with saline 100 μl/mouse (n=6) and random controls with as-hTERT 100 μg/mouse (n=6), and tumor progression was measured daily.

Group III: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla. One day postinjection, each mouse was injected as-hTERT 100 μg/mouse (n=12), saline 100 μl/mouse (n=8) and random controls with as-hTERT 100 μg/mouse (n=8) at the same axilla. Three weeks postinjection, no tumors were detected in 10 animals treated with as-hTERT. These mice were divided further into another two teams: one team (n=6) was injected subcutaneously as-hTERT at the dosage of 30 μg/mouse at the same place as the first time, the other (n=4) was given saline.

In each group, tumor progression was measured, and observations on animal behavior were recorded daily during the study period.

HBV antigen detection

According to the protocol of antigen detection kit (Lizhu Co. Shenzhen, China), the concentrations of HBsAg and HBeAg in the supernatant were detected using an ELISA microtiter plate reader. The results were illustrated with P/N value (P/N=sample A/negative control A. A stands for the amount of light absorbance).

Statistical analysis

All the data were expressed as the mean ± standard error of the mean (SE). The P values were calculated by ANOVA or exact probability method. A P value less than 0.05 was considered statistically significant.

RESULTS

as-hTERT on activity of telomerase

The results were shown with the value of A_{450}nm. A_{450}nm of as-hTERT treated cells, random sequence control, positive control were 0.42, 1.49 and 1.51 respectively (Figure 1). That of the negative control was 0.08. Telomerase activity was inhibited with the addition of 17-base as-hTERT at a concentration of 10 uM.

Antiproliferation effect of as-hTERT on HepG2.2.15 cells

Seventy-two hours after incubation with the drugs, as-hTERT caused significant (F=251.13, P=0.0001) inhibition of cell growth as shown in Figure 1, but not in random sequence controls and saline controls.

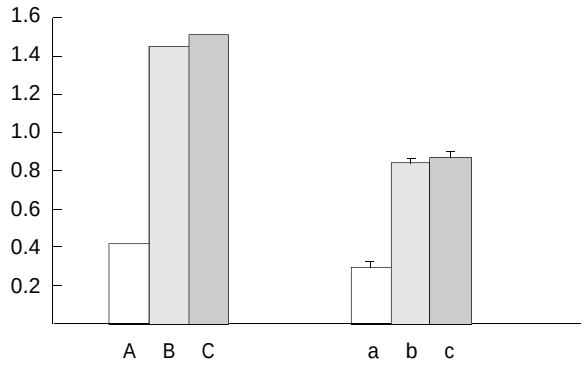

Figure 1 Inhibition effects on HepG2.2.15 cells of as-hTERT *in vitro*. A, B and C: Growth inhibition by MTT method. a, b, and c: Inhibitory effects on telomerase activity by PCR-ELISA. A and a: as-hTERT treated cells, B and b: Random sequence treated cells, C and c: Saline treated cells.

Growth arrest of human hepatocarcinoma in vivo

The drugs were injected into the tumors. After seven days, the peaks of apoptosis of tumor cells treated with as-hTERT, saline control were 21.12% and 7.92% respectively (Figure 2). Inflammation was observed in tissues treated with as-hTERT, but not in controls. The conformation of the animals' liver was normal.

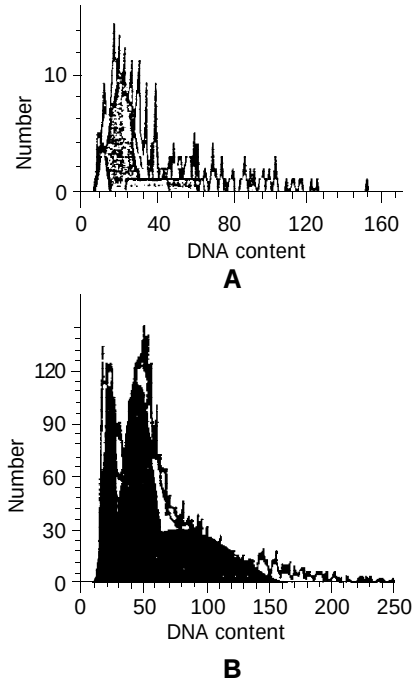

Figure 2 Cell cycle of tumor cells treated with as-hTERT/saline *in vivo*. A: Cell cycle of tumor cells treated with saline. The apoptosis peak was 7.92%(#). B: Cell cycle of tumor cells treated with as-hTERT. The apoptosis peak was 21.12%(*).

As-hTERT could retard tumor cell growth. In the group in which the drugs were injected in combination with HepG2.2.15 cells, tumors were undetectable subcutaneously during the whole study period (10 weeks, 0%, 0/7), while the saline and random sequence controls failed to inhibit tumor growth. In two weeks, tumors were developed in all of the animals in control teams (100%, 8/8). There was a significant difference between as-hTERT treated and control animals (P<0.01).

If the drugs were injected 24 h after the animals were injected with the cells, in three weeks, tumors were detectable in all of the animals in control teams (100%, 8/8), while in team of animals treated with as-hTERT tumors began to appear in two (16.7%, 2/12), and grew very slowly. Furthermore, if the animals without tumor were injected supplementary drugs, subcutaneous tumor was undetectable during the later 6w (0%, 0/6), while in the team of animals without extra addition of the drugs, tumors developed (Figure 3).

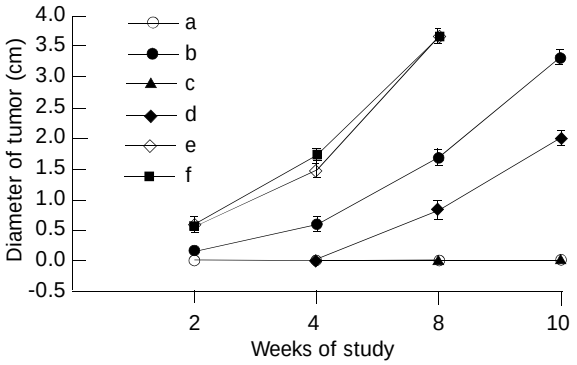

Figure 3 Inhibition effects of tumor cells *in vitro*. a: as-hTERT and HepG2.2.15 cells were injected into animals together. There was no tumor growth in ten weeks. So the diameter of tumor was 0 during the whole study period. b: as-hTERT was injected 24 hours postinjection of the cells. Tumor was not grown in animals which were treated with as-hTERT supplementary (c), otherwise (d), tumors grew again, but very slowly. In random (e) and saline control (f), there was no retarding effect on tumorcell growth. Tumors grew very fast, and after eight weeks, the animals died naturally.

Alteration of HBV antigen expression

as-hTERT could inhibit HBV expression *in vitro* and *in vivo*. *In vitro*, the inhibition peaks on HBsAg and HBeAg were 76% and 56% respectively, while *in vivo*, the inhibition peaks were 38% and 73%.

DISCUSSION

Hepatocarcinogenesis is a very complicated event, and many factors were involved in this process, such as activation of oncogenes, inactivation of tumor suppressor genes and hepatitis virus infection[27]. But the most important is the activation of telomerase. Therefore, to inhibit telomerase activity so as to arrest tumor cell growth by antisense technique is of great significance. In most researches, the target gene was hTR, which is the replication template for telomere, but not the regulatory region of hTERT, the most important region which regulates the activity of tolemerase.

Recent studies suggested that hTERT gene expression was controlled mainly at the transcriptional level[28]. Many cis-elements and trans-acting factors that regulate the hTERT gene transcription have been identified[29-31].Among the potential transcription factor binding sites within hTERT promoter region, a typical E-box (CACGTG, -178--182) which belongs to the bHLHZ family of transcription factors (such as oncogene c-myc), was likely to play an important role in hTERT gene transcription[32-38]. Our results showed that as-hTERT targeting at the binding site of E-box in hTERT promoter region could inhibit the growth of HepG2.2.15 cells *in vitro*. The probable reason was that the regulating effect of E-box on hTERT transcription was eliminated due to as-hTERT complementary

to E-box binding site. Therefore, telomerase activity was decreased, telomere was shortened to the crisis point, and cell growth was arrested, and apoptosis was increased. However, asON could not completely inhibit telomerase activity. This result indicated that E-box is one of the cis-elements which regulate telomerase transcription. Furthermore, it is very important to study on cis-element and transcriptional factors regulating telomerase activity.

AsON targeting at the promoter region of telomerase could inhibit hepatoma cell growth of mice model *in vivo*. Different ways of drugs administration demonstrated different growth inhibitory effects. Apoptosis was detected in tumors injected as-hTERT, but not in cells treated with saline controls. The best growth inhibitory effect on tumor cells was observed in animals injected as-hTERT in combination with the cells. In another team, tumor growth was observed in the mice which were given as-hTERT 24 hours postinjection of HepG2.2.15 cells at the same site, but the tumors grew very slowly. It could not inhibit tumor cell growth completely through this way. However, if the mice were given a smaller dosage of the drug continually, tumor cell growth would be inhibited completely. These results demonstrated that as-hTERT could inhibit tumor cell growth, the earlier the drug were given, the better the effect was. It suggests that as-hTERT can be an effective drug for eliminating the remaining tumor cells after operation.

HBV genome could integrate into the telomerase gene[32,39]. HBV was a potential cis-activation factor which regulated telomerase activity[40]. The expression of HBV gene could increase telomerase activity[41]. This study demonstrated that as-hTERT could affect the expression of HBV antigen both *in vitro* and *in vivo*. The reason may be that as-hTERT inhibited telomerase activity, and cell growth was retarded. Therefore, HBsAg and HBeAg expressions were inhibited. Maybe, there is some relationship between telomerase activity and HBV, which needs to be further clarified.

Furthermore, there was no toxicity on the mice. In summary, we have demonstrated that a short asON which targets at the initiator of hTERT is capable of inhibiting telomerase activity and retarding tumor cell growth both *in vitro* and *in vivo* in a sequence-dependent manner. These results provide further evidence that drugs targeting at telomerase have a therapeutic potential.

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• LIVER CANCER •

Nuclear and mitochondrial DNA microsatellite instability in Chinese hepatocellular carcinoma

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Abstract

AIM: To study the nuclear microsatellite instability (nMSI) at BAT26 and mitochondral microsalellite instability (mtMSI) in the occurrence and development of hepatocellular carcinoma and the relationship between nMSI and mtMSI.

METHODS: nMSI was observed with PCR and mtMSI with PCR-SSCP in 52 cases of hepatocellular carcinoma.

RESULTS: mtMSI was detected in 11 out of the 52 cases of hepatocellular carcinoma (21.2%). Among the 11 cases of hepatocellular carcinoma with mtMSI, 7 occured in one locus and 4 in 2 loci. The frequency of mtMSI in the 52 cases of hepatocellular carcinoma showed no correlation to sex, age, infection of hepatitis B, liver cirrhosis as well as positive AFP of the patients ($P>0.05$). In addition, nMSI was detected in 3 out of 52 cases of hepatocellular carcinoma (5.8%) and there was no correlation of the incidence of mtMSI to that of nMSI ($P>0.05$).

CONCLUSION: mtMSI may be involved in the coccurrence and development of hepatocellular carcinoma and it is independent of nMSI.

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INTRODUCTION

Mitochondria are the energy-transducting organelles of eukaryotic cells in which fuels to drive cellular metabolism are converted into cellular adenosine triphosphate (ATP) through the process of oxidative phosphorylation. Mitochondria are responsible for generating approximately 90% of ATP. The mitochondrion is the only organelle in the cell, aside from the nucleus, which contains its own genome and genetic machinery[1]. Mitochondrial DNA (mtDNA) is a 16 569 base-pair, double-stranded and closed circular molecule, and encodes 13 polypeptides. All of the polypeptides are components of the respiratory chain/OXPHOS system, plus 24 genes, specifying two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs), which are required to synthesize the 13 polypeptides. Mitochondrial genome is far more vulnerable to oxidative damage and undergoes a higher rate of mutation than nuclear genome due to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain, which constantly generates superoxide radicals[2-5]. Accumulation of mutations in mtDNA is approximately tenfold greater than that in nuclear DNA[6].

A high frequency of mtDNA mutations has been identified in cancer of the colon[7], stomach[8], liver[9], pancreas[10], lung[11], breast[12], kidney[13], prostate[14], ovary[15], Barrett' s esophagus[16] and leukemia[17]. The majority of these somatic mutations were homoplasmic, suggesting that mutant mtDNA becomes dominant in tumor cells. In addition, microsatellite instability has also been shown in mtDNA of colorectal and gastric carcinomas[18,19]. Further studies demonstrated that mononucleotide could repeat alteration, missense mutation, and small deletion in NADH dehydrogenase genes and alteration in a polycytidine (C)n tract in the D-loop region of mtDNA could occur in colorectal carcinomas[20]. These results imply that microsatellite instability in mtDNA (mtMSI) of colorectal carcinoma may be resulted from certain deficiencies in DNA repair. Therefore, it has been proposed that somatic mutations and mtMSI play a role in tumorigenesis and development of cancer[21].

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer related mortality worldwide. The incidence of HCC shows a considerable geographical variation with a very high incidence in China. Epidemiological studies in high-risk populations have identified chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infection as well as dietary exposure to aflatoxin B1 (AFB1) as major factors in the etiology of this disease[22]. It has been reported that the amount of AFB1 combined to hepatocellular mtDNA is 3-4 fold larger than that combined to nuclear DNA(nDNA). This combined product of aflatoxin cannot easily be expelled and stays in mtDNA for a long period[22]. Since there is a prolonged period between initial HBV and HCV infection and emergence of HCC, multiple genetic events may occur to promote the malignant transformation of hepatocytes. Many chromosomal aberrations have been frequently reported in HCCs including loss of heterozygosity (LOH) at numerous loci[23,24]. The repeated destruction and regeneration of liver tissue associated with chronic viral hepatitis would lead to accumulation of mtDNA mutations[25]. Although MSI in nuclear DNA (nDNA) of HCCs has been detected[26-32], little attention has been paid to MSI in mtDNA(mtMSI) in this tumor. In order to elucidate the role of mtMSI in the hepatocarcinogenesis, we examined mtMSI and nMSI in a set of 52 Chinese HCCs.

MATERIALS AND METHODS

Fresh tissues were collected from 52 HCC patients undergoing hepatic resection in the Southwest Hospital, Third Military Medical University, Chongqing, China from 1996 to 2002. Neoplastic and nonneoplastic liver tissues were frozen in liquid nitrogen immediately and kept at -70 ℃ until processing. The 52 patients consisted of 42 males and 10 females, their age ranged from 22 to 71 years with an average of 48.8 years at

Table 1 Sequences of primer for PCR analysis

Repeat sequence	mtDNA region	Position	Annealing (℃)	Primer(5'-3')
(C)*n*	270-425	D-loop	58	TCCACACAGACATCAATAACA
				AAAGTGCATACCGCCAAAAG
(CA)*n*	467-556	D-loop	55	CCCATACTACTAATCTCATCAA
				TTTGGTTGGTTCGGGGTATG
(C)6	3529-3617	ND1	55	CCGACCTTAGCTCTCACCAT
				AATAGGAGGCCTAGGTTGAG
(A)7	4555-4644	ND2	55	CCTGAGTAGGCCTAGAAATAAA
				ACTTGATGGCAGCTTCTGTG
(T)7	9431-9526	COIII	55	CCAAAAAGGCCTTCGATACG
				GCTAGGCTGGAGTGGTAAAA
(C)6 and (A)8	12360-12465	ND5	55	CACCCTAACCCTGACTTCC
				GGTGGATGCGACAATGGATT
(CCT)3 and (AGC)3	12940-13032	ND5	55	GCCCTTCTAAACGCTAATCC
				TCAGGGGTGGAGACCTAATT

diagnosis. Thirty-two patients were positive and 20 were negative for hepatitis B surface antigen (HBsAg). Hepatitis C virus antibody (Anti-HCV) was negative for all cases. Hematoxylin and eosin-stained sections were prepared from the same samples used for mtMSI and nMSI studies and the diagnosis of HCC was confirmed by histology. None of the patients included in the present series had a family history suggestive of HNPCC and none had received previous chemotherapy or radiation therapy. Necrotic tumors were excluded from the study. The tumor samples contained more than 70% malignant cells. Genomic DNA was isolated from tumor and non-tumor liver tissues and blood, using standard proteinase-K digestion and phenol-chloroform extraction protocols.

PCR-single strand conformation polymophism (PCR-SSCP) was performed to amplify the microsatellite sequence of mtDNA using published primers[18]. The primer consisted of 2 D-loop regions and 5 coding regions (Table 1). The reaction conditions and procedures were similar to those reported by Hebano *et al*[18].

Each PCR was digested by appropriate restriction enzymes and electrophoresed at 300V at 22℃ for 2 hr on a 7.5% polyacrylamide gel containing 50 mmol/L boric acid, 1 mmol/L EDTA and 2.5% glycerol. After silver staining, PCR products showing mobility shifts were directly sequenced using appropriate internal primer and analyzed using 373A automated DNA sequencer (Perkin Elmer Cetus). All analyses were performed twice to rule out PCR artifact.

MSI at BAT26 microsatellite locus was analyzed using PCR method. The sequence of upper stream primer was 5'-TGACTACTTTTGACTTCAGCC-3' and that of down stream primer was 5'-AACCATTCA ACA TTT TTA ACC C-3'. PCR was performed in 20 μl of reaction mixture containing 10 mmol/L Tris-HCl (pH8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L each deoxyncleotide triphoshate, 0.5 μmol/L of each primer, 0.5 unit Ampli Taq polymerase (Perkin-Elmer Cetus, NowalK), 100 ng genomic DNA and 0.5 μCi [^{33}p] dATP. The reaction was carried out in a thermal cycler at 94 ℃ for 1 min, at 55 ℃-62 ℃ for 1 min, and at 72 ℃ for 1min, for 35 cycles with an initial denaturation step at 94 ℃ for 5 min and final extension step at 72 ℃ for 10 min. The PCR products were then separated on 5% polyacrylamide 7M urea denaturing gel, and visulized by autoradiography. MSI was defined as the presence of a band shift in the tumor DNA not present in the corresponding normal DNA.

χ^2 test was used for statistical analysis and $P<0.05$ was considered as statistically significant.

RESULTS

Fifty-two HCC samples were screened for mtMSI at seven repeat sites using the PCR-RFLP method. Figure 1 exhibits a representative mobility-shift band compared with normal counterpart. mtMSI affecting at least one locus was observed in 11 out of 52 cases (21.2%), in which 7 cases affected 1 locus and 4 cases affected 2 loci. mtMSI occurred in D-loop in 10 cases (19.2%), in which 8 cases occurred in $(C)_n$ region and 2 cases in $(CA)_n$ region. mtMSI occurred in the coding region in 5 cases (9.6%), and concomitant mtMSI locus was found in the D-loop in 4 out of the 5 cases. The frequency of mtMSI in 52 cases of HCC showed no correlation to sex, age, HBV infection, liver cirrhosis and positive AFP of the patients ($P>0.05$, Table 2).

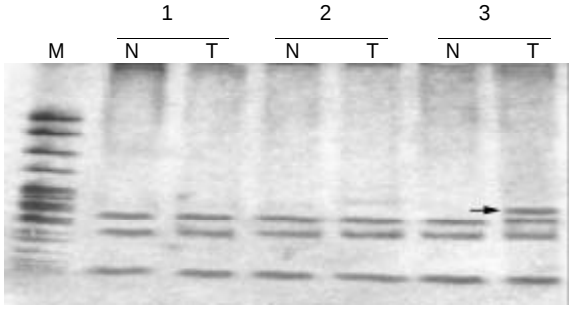

Figure 1 mtMSI in hepatocellular cancer. Arrows indicate conformational variants associated with mtMSI, N: normal DNA, T: tumor DNA.

Table 2 Relationship between MSI and clinical parameters

		n	mtMSI positive	mtMSI negative
Sex	Male	42	10	32
	Female	10	1	9
Age	<30	1	1	0
	30-60	41	8	33
	⩾60	10	2	8
HBsAg	Positive	38	8	30
	Negative	14	3	11
Cirrhosis	Positive	37	9	28
	Negative	15	2	13
AFP	Positive	26	6	20
	Negative	26	5	21

The mobility shift in tumor DNA compared to corresponding normal DNA samples representing nMSI is shown in Figure 2. nMSI was found in 3 of 52 cases of HCC (5.8%). In the 3 cases of nMSI, only 1 case showed mtMSI simultaneously. No correlation was found between nMSI and mtMSI in the 52 cases of HCC.

Figure 2 MSI at BAT-26 in hepatocellular cancer. Arrows indicate conformational variants associated with MSI. N: normal DNA, T: tumor DNA.

DISCUSSION

It has been discovered so far that mitochondria are the only organelle to have their own genome and to undergo replication, transcription and translation without dependence on nuclear DNA. They are called as the "25th chromosome of human body". Many diseases have been found to be related to the structural and functional defects of mitochondria and consequently they are known as mitochondrial diseases[33]. mtMSI has been found to be a very common phenomenon accompanying gastric carcinoma, colorectal carcinoma and breast carcinoma and may play an important role in the carcinogenesis of these malignant diseases[12,18-21]. To study the role of mtMSI in liver carcinogenesis, we analyzed 52 cases of HCC using seven microsatellite markers known to be altered in gastrointestinal carcinomas. mtMSI in at least one locus was found in 11 of the 52 cases (21.2%) of HCC, implying that mtMSI might occur not only in gastrointestinal cancers but also in hepatic cancers, and it may play an important role in the occurrence of a certain number of HCC.

Unlike other types of cancer, HCC has been found usually preceded by chronic inflammation due to viral infection[34-37]. Matsuyama *et al*[11] reported that the frequency of mtDNA mutations was markedly increased in both noncancerous and cancerous liver specimens compared with control liver tissue. Accumulation of mtDNA mutations in HCC tissue could reflect its malignant potency. The frequency of mtDNA mutations was significant higher in HBV infection-related HCC than in other tumors, which implies that repeated destruction and regeneration of the liver tissue associated with chronic viral hepatitis would lead to accumulation of mtDNA mutations. In the current study, we did not find any obvious relationship between mtMSI and HBsAg, suggesting that HBV infection might play a limited role in the mtMSI pathway of HCC. In addition, we did not find an obvious relationship between mtMSI and sex, age, cirrhosis as well as positive AFP.

MtDNA contains several mono- and dinucleotide repeats. The most frequently used mtDNA in the test of mtMSI is a (CA)n microsatellite starting at 514 bp position of the D-loop[38] and a homopolymeric C tract extending from 16 184 to 16 193 bp of the D-loop, which could be interrupted by a T at 16 189 bp position[39]. Alonso *et al*[40] studied mutations in the mtDNA D-loop region and found three mutations in eight gastric tumors. Richard *et al*[41] studied 40 pairs of normal/cancer breast specimens for the presence of mtMSI and found a 216-fold increase in the D-loop point mutations of cancer cells with regard to the spontaneous rate detected in female gametes. Maximo *et al*[19] utilized PCR-SSCP to examine mtDNA large deletions and mutations in 32 gastric carcinomas and found that most of the mutations corresponded to insertions/deletions in the D-loop region or transitions in ND1, ND5, and COXI. Earlier studies revealed the presence of mutations in the D-loop of both non-malignant and malignant gastric tumors[42,43]. Analysis of HCCs indicated that mutations in the D-loop were a frequent event and could be used as a molecular tool for the determination of clonality[9,44]. Two recent studies reported the frequency of D-loop mutations in esophageal cancer. One group focused on adenocarcinomas of Barrett' s esophagus. In that study, D-loop alterations were identified in 40% of the patients examined[16]. The other study showed that D-loop mutations were much less frequent in esophageal cancer, occurring in only 5% of the specimens analyzed[45]. Clearly, analysis of mtDNA from more esophageal tumor samples is needed in order to determine the frequency of D-loop mutations and their relevance in this type of cancer. In our series of 52 cases of HCC, mtMSI was found in 11 (21.2%). MtMSI occurred in the D-loop region of 10 cases and in the coding region of 5 cases. Among the 10 cases, mtMSI occurred in (C)n region of 8 cases and in (CA)n region of 2 cases, So (C)n region of D-loop is the site at which mtMSI occurs more frequently than in other regions. Our findings are consistent to those reported by Habano *et al*. and Maximo *et al*[18-20].

Microsatellite markers might provide evidences of faulty DNA mismatch repair (MMR) via the detection of MSI[46-49]. The choice of microsatellite markers may impact on the MSI detection rate. BAT-26, a repeat of 26 deoxyadenosine localized in intron 5 of hMSH2 gene, has been reported as a reliable indicator of replication error phenotype in colorectal cancers, enabling analysis of tumour DNA in the absence of paired normal DNA[50]. The frequency of nMSI in hepatic cancer varied in different reports[51,52]. Karachristos *et al*[52] studied 27 cases of HCC and found none of the tumors examined showed alterations in BAT-26. In our series of 52 cases, 3 cases were found to have nMSI at BAT26 (5.8%).Our finding indicates that nMSI at BAT26 is not common in cases of HCC and support the hypothesis that HCC is a "low" MSI tumor in China. Carcinogenesis of HCC may undergo a different molecular route other than that of nMSI.

Mutation of mtDNA may result in the occurrence of tumor but its mechanism remains unknown. Further studies are required to determine if mtDNA mutations are correlated with malignant transformation. Recently, scholars have shifted their attention to the interactions between mtDNA and nDNA. Fragments of mtDNA are sometimes found in nuclear genes, and the insertion of mtDNA has been suggested as a mechanism by which oncogenes are activated[53]. For example, sequences representing subunits ND4 (Complex I) and subunits cytochrome C oxidases I, II and III (complex IV) have been found in the nuclear DNA of various tissues[53]. In yeast cells, migration of DNA from the mitochondria to the nucleus occurred 100 000 times more frequently than in the opposite direction[54]. In our series of 52 cases of HCC, nMSI was detected in 5.8% and coexistance of nMSI and mtMSI in only 1 out of 3 cases. We failed to confirm there was a correlation of mtMSI to nMSI in our cases of HCC. This finding is in agreement with the recently published data on gastrointestinal cancer[55].

In conclusion, mtMSI could play an important role at multiple stages in the process of carcinogenesis. The mitochondrial production of ROS might be involved in the initiation and promotion of carcinogenesis, in part due to ROS-triggered mutagenesis of both mtDNA and nDNA[56]. Also, other evidences exists for a mechanism of nDNA mutagenesis involving the integration of mtDNA fragments. Many primary tumors revealed a high frequency of mtDNA mutations and

the majority of these somatic mutations were homoplasmic in nature, indicating that the mutant mtDNA has become dominant in tumor cells. The mutated mtDNA was readily detectable in paired bodily fluids from each type of cancer and was 19 to 220 times as abundant as mutated nuclear p53 DNA. By virtue of their clonal nature and high copy number, mitochondrial mutations might provide a powerful molecular marker for noninvasive detection of cancer[57]. Important areas for future research should include intergenomic signaling pathways in carcinogenesis and the potential role of mitochondria and mtDNA mutations in immunological surveillance of tumor cells. Finally, the role of mitochondria in stimulating apoptosis could be exploited in cancer therapeutics[58].

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• LIVER CANCER •

Hemizygous deletion and hypermethylation of RUNX3 gene in hepatocellular carcinoma

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Abstract

AIM: To analyze the genetic and epigenetic alterations of RUNX3 gene, a potential putative tumor suppressor gene, in hepatocellular carcinoma (HCC).

METHODS: PCR-based loss of heterozygosity (LOH) detection, analysis of mutation with PCR-single strand conformational polymorphism (SSCP) and sequencing, and methylation study with methylation specific PCR (MSP) were performed on RUNX3 gene in a series of 62 HCCs along with their matched normal tissues.

RESULTS: Mutation of RUNX3 gene was not found, but one single nucleotide polymorphism with T to A transversion at the second nucleotide of the 18th condon was found. Nine of 26 informative cases (34.6%) showed allelic loss on the polymorphic site and 30 cases (48.4%) revealed hypermethylation of RUNX3 gene in promoter CpG islands. Furthermore, of the 9 cases with LOH, 8 (88.9%) also had hypermethylation.

CONCLUSION: Our findings indicate that inactivation of RUNX3 gene through allelic loss and promoter hypermethylation might be one of the major mechanisms in hepatocellualr carcinogenesis.

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INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional cytokine known to be a potent growth inhibitor for most epithelial cells[1,2]. TGF-β signaling pathway is composed of TGF-β type I, type II receptors and Smad proteins, and is transducted by forming heteromeric complex with its type I and type II transmembrane Ser/Thr kinase receptors. Activated type I receptors then activate the cytoplasmic Smad 2 and Smad 3 by phosphorylation, allowing them to form a hetermeric complex with Smad 4. This Smad complex can activate TGF-beta responsive gene transcription only after it is translocated to nucleus and bound to the specific target nuclear matrix site[2]. However, the key process of nuclear translocation and subnuclear distribution for regulating transcription of TGF-β-responsive gene needs a broad range of nuclear proteins[3]. Recently, RUNX proteins, including RUNX3 gene were proved to interact through their C-terminal segment with Smads and recruit Smads to subnuclear sites of active transcription, thus exerting their biological control[4]. The function of RUNX proteins has been considered as the subnuclear acceptor proteins for signal transduction. On the contrary, Smads cannot be directed to the nuclear matrix in the absence of RUNX proteins[4]. Therefore, TGF-β-Smad signal pathway would be disrupted. RUNX3, one member of the RUNT domain family, was recently found with a loss of 40-60% of expression due to a highly frequency of hemizygous deletion and hypermethylation in gastric cancer[5]. Also, the gastric mucosa of RUNX3 knocked out mouse exhibited hyperplasia and suppressed apoptosis and growth-inhibition induced by TGF-β in epithelial cells[5]. Taken together, it is strongly suggested that RUNX3 gene be a novel tumor-suppressor gene.

Hepatocelluar carcinoma (HCC) is one of the most common causes of cancer death in the world, especially in Asia and Africa[6,7]. HCC, like many other kinds of human malignancy, has been reported to overexpress TGF-β[8]. The serum concentration of TGF-β is also elevated with tumor progression[9]. Therefore, HCC cells resistant to the anti-proliferative function of TGF-β may be a critical step in the development of HCC[10]. However, until the present no molecular event has been found to contribute to the impairment of TGF-β signal pathway in HCC[11]. It is well documented that aberrance of molecules of the pathway including TGF-β receptor, Smads 2, 3, 4, 6 and 7 was very rare in HCC[12-14]. The exact mechanism of HCC with loss of TGF-β responsiveness still remains unknown. A growing body of evidence showed that chromosome 1p36 was a common deletion region where just loci of RUNX3 gene exist[15,16]. Several putative tumor suppressor genes are believed to be in this region. But, different types of tumor have different regions of consensus deletion. For example, the consensus deletion of neuroblastoma has been mapped to 1p36.2-36.3, a region distal to the deleted region in HCC. While in HCC, a minimally deleted region of about 4 Mb on chromosome 1p36 was well defined[17]. Within the common deletion region, another candidate tumor suppressor gene, retinoblastoma protein (Rb)-interacting zinc finger gene (RIZ) was also identified[18]. Unfortunately, mutation of RIZ gene was not found in HCC[15]. Its role in hepatocarcinogenesis has not been clarified yet. Most notably, LOH encompassing RUNX3 gene occurs in early stage of HCC, even in precancerous condition[19,20]. In the current paper, we studied the genetic and epigenetic alterations of RUNX3 gene in HCC in order to find out new clues to the development of HCC.

MATERIALS AND METHODS

Tissue samples

Sixty-two frozen HCC specimens and their adjacent normal liver tissue specimens were obtained from Southwest Hospital, Third Military Medical University, Chongqing, China. Informed consents were obtained from every patient. The patients' age ranged 29-72 years with an average of 48.6 years. The male to female ratio was 52:10. The background liver showed cirrhosis in 53 (85.4%) cases, chronic persistent hepatitis in 6 cases

(9.7%), and non-specific change in 3 (3.2%) cases. HBV was detected in 49 cases (79.0%), HCV was detected in 5 (8.1%) and non-virus hepatitis in 8 (12.8%). The number of cases with histological grades I, II and III was 8, 26, 28, respectively. Three pathologists reviewed independently one 5 μm thick section stained with hematoxylin and eosin.

DNA extraction

Frozen tissue samples were ground into very fine powder in liquid nitrogen, suspended in lysis buffer and treated with proteinase K. DNA was extracted by phenol-chloroform-isoamyl alcohol and ethanol precipitation[21]. Adjacent normal liver tissues were used as corresponding normal controls.

Single strand conformational polymorphism (SSCP) and DNA sequencing

A total of 6 exons of RUNX3 gene were screened for inactivation mutations with PCR-SSCP, cyclic sequencing on genomic DNA templates. The primers were designed with OLIGO software program (version 5.0; National Bioscience Inc., Plymouth. MN) using the genomic sequences obtained from GenBank (accession No. NT_004391). PCR primer pairs for amplification of RUNX3 gene are described in Table 1. Each PCR reaction except for exon 2 was performed under standard conditions in a 10 μl reaction mixture containing 1 μl of template DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCI_2$, 0.5 unit of Taq polymerase (Ampli Taq Gold™ containing antibody to Taq, Roche), 0.5 μCi of ^{32}P-dCTP (Amersham, Buckinghamshire, UK), and 1 μl of 10X buffer. Advantage^R-GC genomic PCR kit (Clontech Laboratories, Inc., CA, USA) was used to amplify exon 2 containing CpG-rich sequence according to the user manual. The reaction mixture was denatured for 5 min at 95 ℃ and incubated for 35 cycles (denaturing for 30 s at 95 ℃, annealing for 30 s at 51-67 ℃, and extending for 30 s at 72 ℃). A final extension was continued for 5 min at 72 ℃ in a thermal cycler (PE 480, USA). After amplification, the PCR products were denatured for 5 min at 95 ℃ at 1:1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH and loaded onto a SSCP gel (FMC mutation detection enhancement system, Intermountain Scientific, Kaysville, UT) with 10% glycerol. After electrophoresis, the gels were transferred to 3-mm Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY). For the detection of mutations, DNAs showing mobility shifts were cut out from the dried gel, and reamplified for 30 cycles using the same primer set. Sequencing kit (Perkin-Elmer, Foster City, CA) was used according to the manufacturer' s recommendations. Cycling sequencing products were resolved on a 6% denatured sequencing gel (USB™, Cleveland, USA).

Loss of heterozygosity (LOH) analysis

We found 1 polymorphic site during SSCP and sequencing analysis using the primer sets covering 6 exons. The polymorphic site had highly frequent information of heterozygote in HCC patients. This made it feasible as an intragenic polymorphic marker for LOH analysis of RUNX3 gene. PCR and SSCP conditions for LOH analysis were exactly the same as described above. PCR products from the corresponding normal and tumor DNAs were run on SSCP gel. Allelic loss was scored when the band intensity of one allelic marker was significantly decreased (more than 70% reduction) in tumor DNA as compared with that in normal DNA.

DNA methylation analysis of RUNX3 gene by methylation specific PCR (MSP)

The methylation status of RUNX3 gene was determined by sodium bisulfate treatment of DNA followed by methylation-specific polymerase chain reaction (MSP), as described with modification[22,23]. In brief, about 100 ng DNA was incubated in 0.2 M NaOH at 42 ℃ for 30 minutes in a total volume of 50 μl. After the addition of 350 μl of 3.6 M sodium bisulfate (Sigma) containing 1 mM hydroquinone at pH 5, the samples were incubated for 4-5 hours at 55 ℃ in the dark. The modified DNA was recovered with 5 μl of glassmilk (BIO 101, Inc., CA, USA) and 800 μl of 6 M NaI. The glassmilk catching the modified DNA was washed three times with 70% ethanol at room temperature, and then treated with 0.3 M NaOH/90% ethanol once, washed twice again with 90% ethanol. The DNA was finally eluted from the dried pellet with 30 μl of 1 mM Tris-HCI (pH 8.0) for 15 minutes at 55 ℃. Five μL of bisulfate-modified DNA was subjected to MSP using two sets of primer specific for methylation detection and unmethylation detection as reported previously[5]. PCR was performed in a total volume of 30 μl containing 5 μl template DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCI_2$, 0.5 unit of Taq polymerase (Ampli Taq Gold™ containing antibody to Taq, Roche) and 3 μl of 10X buffer. The reaction solution was initially denatured at 95 ℃ for 1 minute. Amplification was carried out for 40 cycles at 95 ℃ for 30 s, at 63 ℃ for 30 s and at 72 ℃ for 30 s, followed by a final extension at 72 ℃ for 5 min. Controls without DNA were performed for each set for PCRs. Ten μl of PCR products was directly loaded onto 2% agarose gel containing ethidium bromide, and directly visualized under UV illumination, and photographed. The size of PCR products was 234 bp.

RESULTS

Frequency of LOH in RUNX3 gene and its clinical significance

We failed to detect a mutation in all six exons and partial intron adjacent to exon in 62 HCCs by PCR-SSCP and sequencing. But the polymorphic site with T to A transition at the second nucleotide of codon 18 was found at exon 1, a relatively high frequency of heterozygotes (26/62) was used as an intragenic marker to examine LOH of RUNX3 gene, and 34.6% (9/26) of informative cases showed allelic loss (Figure 1).

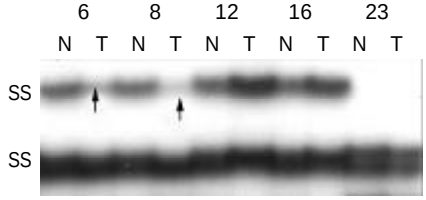

Figure 1 Allelic loss of RUNX3 gene in HCC. N: normal, T: tumor, Arrow indicate allelic loss.

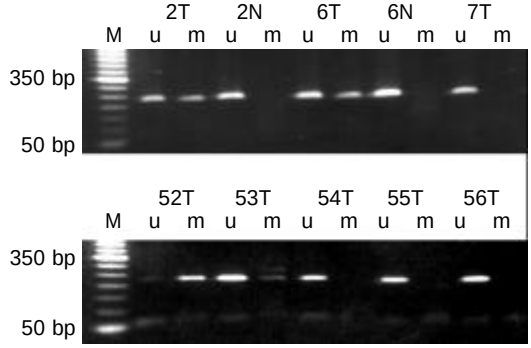

Figure 2 Methylation state of RUNX3 gene in HCC. M: Molecular weight of 50 bp DNA ladder, u: unmethylation, m: methylation, N: normal, T: tumor.

Table 1 Primers used for PCR amplification of RUNX3 gene

Name	Exon	Primer sequence	Annealing Tm(℃)	Size (bp)
RUNX3_E1	1	F:5'-CTGGCCACAGCTCCCCACC-3'	64	158
		R:5'-ATCCCAACCCAACCCCCTGAAG-3'		
RUNX3_E2-1	2	F5'-CTGCTTTCCCGCTTCTCGCGGCAGC-3'	62	212
		R:5'-CGCCGCTGTTCTCGCCCATCTTGCC-3'		
RUNX3_E2-2	2	F:5'-CCAAGCACCAGCCGCCGCTTCACAC-3'	65	207
		R:5'-GAGGAAGTTGGGGCTGTCGGTGCGC-3'		
RUNX3_E2-3	2	F:5'-CTCGATGGTGGACGTGCTGGCGGAC-3'	62	230
		R:5'-GTCTCGGGCACCTCCCATCCCCACT-3'		
RUNX3_E3	3	F:5'-TGCCATTGCCAATGCTGAA-3'	58	240
		R:5'-TAAGCTGTCCCCCTGCATCC-3'		
RUNX3_E4	4	F:5'-GCACTGGACCCTCCTCCC-3'	60	187
		R:5'-CACCTGCCTCTATTCCCCACT-3'		
RUNX3_E5	5	F:5'-CGCTGCAGCCCCTCCCTC-3'	65	225
		R:5'-GGGGAAGCAACGGCTGATGG-3'		
RUNX3_E6-1	6	F:5'-GTTGTTAGGGTCCCCGCCTCC-3'	63	224
		R:5'-CTACCCGCATGCTGGCCTCTT-3'		
RUNX3_E6-2	6	F:5'-CGCTGCTGGTGCAAGAGG-3'	63	205
		R:5'-ACCAGCCGCTTCCACCATAC-3'		
RUNX3_E6-3	6	F:5'-TGGCGGGAGGTAGGTATGGTG-3'	63	166
		R:5'-GCCAACCCTCACGGAGAGC-3'		
RUNX3_E6-4	6	F:5'-AATGCATCCTGGGGTCTGG-3'	61	150
		R:5'-GGGTTGTCTCGAGCTGGAACT-3'		

Frequent hypermethylation of RUNX3 gene in HCC

On the basis of the presence of a CpG island in the 5' region of RUNX3 gene, we examined the promoter 2 hypermethylation using two sets of primers specific for MSP reported by Li[5], and 48.4% (30/62) HCCs were found to have hypermethylation (Figure 2). Notably, we found the degree of hypermethylation was quite different among individual tumors by comparing with the intensity between unmethylation and methylation bands under the same PCR conditions. Although our methods could not be used to quantitate methylation, hypermethylation was not found in the matching normal liver tissues.

Biallelic aberrant of RUNX3 gene in HCC

LOH and hypermethylation are two distinct ways to inactivate tumor suppressor gene. It is widely known that both of them were often involved in complete loss of gene function through cooperation. We, here, found 8 cases had hypermethylation in the 9 cases with LOH of RUNX3 gene.

DISCUSSION

RUNX3 gene has been found belonging to the runt domain family of transcription factors acting as master regulators of gene expression in major developmental pathway[3]. At present, three RUNX genes, RUNX1, RUNX2 and RUNX3, have been identified. All the three genes have been found to share a highly conserved region, called runt domain[24]. They have been shown to interact with Smads 1, 2, 3 and 5, and are indispensable in mediating Smads compound nuclear distribution and Smads specific binding to target DNA[25,4]. Therefore, RUNX proteins are important targets of TGF-beta-Smads signaling pathway. It is well documented that mutation of RUNX1 gene was associated with the development of acute myelogenous leukemia, while mutation of RUNX2 contributed to celeidocranial dysplasia (CCD)[25]. Recently, RUNX3 gene was found to play an important role during the development of gastric cancer. Its absence would lead to abnormal proliferation of gastric epithelial cells, lack of responsiveness to apoptosis and growth-inhibitory effect induced by TGF-beta in knock out mouse. Moreover, wild type RUNX3 gene significantly reduced the tumorigenesis ability of tumor cells, whereas mutant type RUNX3 gene would abolish the tumor suppressor action of RUNX3 in nude mice and drive tumor cells to grow much faster. In human primary gastric tumor, 60% cases do not significantly express RUNX3 gene because of hemizygous deletion and hypermethylation. Correlation between hypermethylation and under-expression or no expression was further confirmed in *in vitro* experiment[5]. These evidences strongly suggest that RUNX3 gene be a tumor suppressor gene.

It is commonly known that TGF-beta-Smad signal pathway is disrupted in HCC, but the exact mechanism of disruption of the signal pathway has still remained to be worked out[8]. Furthermore, LOH of 1p36 encompassing RUNX3 gene was a common event in pathogenesis of HCC[19]. So, it is reasonable to consider RUNX3 gene as a most possible target gene in the development of HCC. In this study, we found 34.6% of HCC showed LOH of RUNX3. This result is in concordance with the previously reported 30% more or less frequency of LOH at 1p36, an early event in the development of HCC[17,20]. Unfortunately, no mutation was discovered in 62 HCCs.

Hypermethylation is a regional event that occurs frequently in GC-rich sequences, called CpG islands, often located within the 5' regulatory regions of non-transcribed genes. In contrast, actively transcribed genes are always in unmethylation status. Inactivation of genes by hypermethylation of their CpG islands has been well clarified[21,26,27]. Now, it has been recognized that hypermethylation of CpG islands in the promoter region is an alternative way to silence some cancer-associated gene as effectively as inactivation by mutation or deletion[28,29]. To date, genes involved in regulation of cell cycle[21,30], DNA repair[31],

angiogenesis[32], and apoptosis[33,34] have been shown to be inactivated by hypermethylation which is also a frequent event in many human cancers including hepatocellular carcinoma[35-37]. For RUNX3 gene, transcription is regulated by two distinct promoters, P1 and P2. The major RUNX3 mRNA is transcribed from P2. The genomic region surrounding the P2 promoter constituted a large (4.2 kb) CpG island with a GC content of 64%[24]. These features showed P2 possessed the hallmark characteristics of GC-rich promoters. So, it is rational that transcription from P2 should be regulated by DNA methylation in theory. In practice, Li *et al*[5] confirmed the presumption in *in vitro* experiment. Our analysis of RUNX3 gene in HCCs provided evidence of promoter hypermethylation, a common alteration as well as an early event. Thus, methylation of the promoter region appears to be the dominant mode of inactivation of RUNX3 gene in human HCC, just the same as in human gastric cancer. Unfortunately, we did not detect the expression of RUNX3 gene due to unavailability of the sample and antibody. However, methylation changes are considered as a surrogate for altered expression of the gene product, thus, the detection of any abnormally methylated site is a strong indication that this mechanism could alter the expression levels of target genes. It was reported that hypermethylation correlated with LOH and often occurred before the allelic loss[38]. We found in our study 48.3%(30/62) of HCCs showed hypermethylation, which was higher than LOH (34.6%), and 88.9%(8/9) of HCCs with LOH had hypermethylation in the promoter of RUNX3 gene, hence being in line with Knudson' s two hit hypothesis and consistent with previous reports.

In summary, we have demonstrated a high frequency of RUNX3 gene aberration-allelic loss together with hypermethylation of the remaining alleles in HCC. These observations can provide the evidence that promoter hypermethylation and allelic loss are the major mechanisms for inactivation of RUNX3 gene in HCC. RUNX3 gene may be one of the key tumor suppressor genes at 1p36 which is the common deletion site of RUNX3 gene in HCC. Inactivation of RUNX3 gene functions, resulting in impairment of TGF-beta - Smads signal pathway and other tumor suppressor function, may be closely associated with the development of HCC.

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• VIRAL HEPATITIS •

Evaluation of high-resolution computed tomography and pulmonary function tests in patients with chronic hepatitis C virus infection

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Abstract

AIM: To investigate pulmonary involvement via pulmonary function tests (PFT) and high-resolution computed tomography (HRCT) in patients with chronic hepatitis C virus (HCV) infection.

METHODS: Thirty-four patients with chronic HCV infection without diagnosis of any pulmonary diseases and 10 healthy cases were enrolled in the study. PFT and HRCT were performed in all cases.

RESULTS: A decrease lower than 80% of the predicted value was detected in vital capacity in 9/34 patients, in forced expiratory volume in one second in 8/34 patients, and in forced expiratory flow 25-75 in 15/34 patients, respectively. Carbon monoxide diffusing capacity (DLCO) was decreased in 26/34 patients. Findings of interstitial pulmonary involvement were detected in the HRCT of 16/34 patients. Significant difference was found between controls and patients with HCV infection in findings of HRCT (χ^2=4.7, P =0.003). Knodell histological activity index (KHAI) of 28/34 patients in whom liver biopsy was applied was 9.0±4.7. HRCT findings, PFT values and DLCO were not affected by KHAI in patients with HCV infection. In these patients, all the parameters were related with age.

CONCLUSION: We suggest that chronic hepatitis C virus infection may cause pulmonary interstitial involvement without evident respiratory symptoms.

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INTRODUCTION

Hepatitis C virus (HCV) is a common infectious agent, and it is estimated that 3% of the world population are infected with HCV. It was reported that HCV caused 20% of acute hepatitis and 70% of chronic hepatitis[1,2]. HCV could be stimulated chronically by immune system[3,4]. There are few studies about pulmonary involvement of chronic HCV infection. These have been done with small patient groups and results on the association between chronic HCV infection and pulmonary involvement could not be found from these studies[5,6].

Although idiopathic pulmonary fibrosis is considered to be idiopathic, inhaled substances are suggested to be responsible for the manifestation of this clinical presentation[7,8]. Onset of symptoms following a viral infection or common cold in some patients suggests that development of the disease may be due to the injury related to the infection. There is evidence that hepatitis C virus, Epstein-Barr virus (EBV), and adenoviruses may be responsible for the fibrosis[9-11].

This study was to investigate the relationship between HCV infection and interstitial pulmonary involvement, and to reveal the relationship among involvement and age, sex, cigarette smoking, severity of hepatitis, and respiratory functions.

MATERIALS AND METHODS

Patients and study design

Thirty-four patients with chronic HCV infection at outpatient clinics of our hospital, were included in the study. Written informed consent was obtained from each patient prior to participation. Ten healthy subjects (6 males, 4 females) were enrolled in the study as control group. Their age, sex characteristics, and smoking habit were similar to the patient group.

Diagnosis of chronic HCV infection was established by the 3rd generation ELISA test (AxSYM HCV version 3.0, Abbott, Wiesbaden-Delkenheim, Germany) and liver biopsy. When liver biopsy was impossible (6 cases), HCV RNA positivity was accepted for the diagnosis.

Patients with previous diagnosis of another pulmonary disease, decompensated cirrhosis, congestive heart failure, suspected malignancy, collagen tissue disease, leukopoenia (<3 000/mm^3), thrombocytopenia (<80 000/mm^3), hepatitis B carriers and drug addicts (current and past users) were excluded. Cases having a history of chronic alcoholism were also not included in the study. All the patients did not receive any treatment for HCV infection.

Medical history was recorded and physical examination was performed in cases who fulfilled the above criteria for study entry. Their age, sex and quantity of cigarette smoking (pack-year) were recorded. Chest X-ray was obtained.

Pulmonary function test (PFT), diffusion test and high-resolution computerized tomography (HRCT) were performed in all cases.

Pulmonary function test

Measurements of vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in first second (FEV1), forced mid-expiratory flow rate (FEF25-75), carbon monoxide diffusion capacity (DLCO) and ratio of DLCO to alveolar ventilation (DLCO/VA) were done in accordance with American Thoracic Society criteria[13] by using the Vmax 22 device (Sensor Medics, Yorba Linda, CA, USA) with single breath diffusion method. None of the patients had received bronchodilator drugs prior to the tests. Measurements were

recorded as the percentage of the predicted value, 80% and above were considered as normal.

High resolution computerized tomography

HRCT images were obtained by the Somatom DRH device (Siemens, Erlangen, Germany), without contrast administration, and with 10 mm interval, 2 mm thick section, 310 mAs, 125 kVp, 4 seconds of imaging time, in bone algorithm, 512x512 reconstruction matrix, and 1 600/-400 parenchymal and 350/50 mediastinal window range.

For evaluation of interstitial involvement with HRCT, the method described by Remy-Jardin *et al*[13] was used. HRCT scans were evaluated for the presence, distribution, and extent of the following signs: [a] ground-glass attenuation, [b] nodular areas of high attenuation, [c] consolidation, [d] linear areas of high attenuation, classified as nonseptal lines, [e] septal lines, [f] honeycombing, and [g] architectural distortion.

Extension of the involvement was assessed independently for each of the three zones of the thorax defined as follows. The upper zones were above the level of the main carina, the middle zones were between the level of the main carina and the inferior pulmonary veins, and the lower zones were under the level of the inferior pulmonary veins. HRCT scores in the upper, middle, and lower pulmonary zones were determined by visually estimating the extent of the disease in each zone. The HRCT score was based on the percentage of pulmonary parenchyma that showed evidence of each recorded abnormality, and was estimated to be 5% of parenchymal involvement: 25% and below as 1 point, 26%-50% as 2 points, 51%-75% as 3 points, 76% and above as 4 points. The scores for each zone were then added to obtain a global extent score, ranging from 0 to 12, and referred to as the HRCT extent score of each HRCT abnormality. A total score of pulmonary involvement was obtained by summation of the global extent score of all HRCT abnormalities, ranging from 0 to 84, which was the feature referred to as the overall HRCT of disease severity.

Pulmonary interstitial involvement was confirmed with prone position scanning in patients who had HRCT findings. HRCT scans were interpreted in random order by two radiologists without any clinical data, the two observers assessed the scans together to reach a decision by consensus.

Evaluation of liver biopsy

Liver biopsy was performed in 28/34 patients. Histological evaluation of liver biopsy reflecting the activity level of the disease was done according to the Knodell histological activity index (KHAI) described by Knodell *et al*[14]. Inflammation in portal areas, piecemeal appearance and bridging necrosis at periportal areas and necro-inflammatory activity observed in parenchyma were considered in the evaluation of the severity of hepatitis.

Statistical analysis

Quantitative data were presented as mean±SD. Statistical analysis was performed by Mann-Whitney *U* test for comparison of PFTs of patients with HCV infection and control group, and Fisher' s exact (chi-square) test for comparison of HRCT findings. Relation among KHAI and age, sex was evaluated by multiple linear regression analysis. HRCT and PFT were considered as dependent variables, KHAI and factors possibly affecting pulmonary pathologies including age, sex and amount of cigarette smoking were considered as independent variables, then separate multiple linear regression analyses for HRCT score, VC, FVC, FEV1, FEF25-75, DLCO, DLCO/VA were performed. $P<0.05$ was considered statistically significant.

RESULTS

Thirty-four patients (15 women and 19 men) with a mean age of 47.6±17.5 years (20-72) were enrolled in the study. Eighteen patients had a history of cigarette smoking and the mean amount of cigarette smoking was 16.5±9.7 pack-year. Ten healthy control cases (6 men and 4 women) with a mean age of 46.2±2.6 years (21-65) were enrolled into the study. Four of them had a history of cigarette smoking.

PFT measurements revealed that VC, FVC, FEV1, and FEF25-75 were below 80% of the predicted value in 9/34, 8/34, 5/34 and 15/34 patients with HCV infection, respectively. DLCO was decreased in 26/34 patients and DLCO/VA ratio was decreased in 18/34 patients. But, there was no significant difference between controls and patients with HCV infection in mean PFT parameters (Table 1). KHAI values were between 2-16 in 28 patients in whom liver biopsy was performed and the mean KHAI was 9.0±4.7 points.

Table 1 Knodell histological activity index (KHAI) and pulmonary function tests (mean±SD) in patients with chronic hepatitis C virus infection and controls

Features	Patients with HCV (n=34)	Controls (n=10)	P
KHAI	9.0±4.7	-	-
VC[a]	86.3±10.9	83.6±7.9	NS
FVC[a]	85.8±12.0	80.6±8.3	NS
FEV1[a]	86.5±11.0	82.3±6.7	NS
FEF25-75[a]	78.2±18.8	81.7±13.5	NS
DLCO[a]	66.3±21.3	80.7±15.3	NS
DLCO/VA[a]	77.7±18.0	85.2±14.5	NS

[a]: % predicted; NS: Not significant.

Interstitial pulmonary involvement was found in 16/34 patients with HRCT. Only one case (1/10) had distortion in the controls in HRCT. HRCT findings excluded other causes such as pneumonia, cancer or tuberculosis in patients and control cases. There was a significant difference between controls and patients with HCV infection in HRCT for interstitial involvement (χ^2=4.7, P=0.03) (Table 2). All of our

Table 2 High-resolution computed tomography (HRCT) findings in patients with chronic hepatitis C virus infection and controls

HRCT Findings	Patients with HCV infection			Controls		
	n	%	HRCT scores	n	%	HRCT scores
Ground-glass attenuation	1	2.9	2	0	0	-
Nodular areas of high attenuation	6	17.6	3.6±1.5	0	0	-
Consolidation	0	0	-	0	0	-
Non-septal lines	5	14.7	2.7±1.7	0	0	-
Septal lines	4	11.7	3.9±1.6	0	0	-
Honeycombing	0	0	-	0	0	-
Distortion	0	0	-	1	10	2

P=0.03, χ^2=4.7.

Table 3 Multiple linear regression analysis of Knodell histological activity index (KHAI) and age and sex in patients with chronic hepatitis C virus infection

	Independent variables								
	Whole regression equation			Age			Sex		
Dependent variable	R^2	*F*	*p*	β	*t*	*p*	β	*t*	*p*
KHAI	0.61	38.8	<0.001	0.78	6.2	<0.001	a	a	a

a: Not significant, R^2: squared multiple correlation coefficient, *F: F* value, β: Partial correlation coefficients, *t: t* value of regression coefficients.

Table 4 Multiple linear regression analysis of high-resolution computed tomography (HRCT) score, pulmonary function test, Kn odell histological activity index (KHAI), age, sex and smoking in patients with chronic hepatitis C virus infection

Dependent variables	Independent variables														
	Whole regression equation			KHAI			Age			Sex			Smoking		
	R^2	*F*	*p*	β	*t*	*p*	β	*t*	*p*	β	*t*	*p*	β	*t*	*p*
HRCT score	0.17	5.4	0.028	a	a	a	0.42	2.3	0.028	a	a	a	a	a	a
VC	0.19	6.1	0.020	a	a	a	-0.44	-2.5	0.020	a	a	a	a	a	a
FVC	0.24	8.0	0.009	a	a	a	-0.49	-2.8	0.009	a	a	a	a	a	a
FEV1	0.25	8.6	0.007	a	a	a	-0.50	-2.9	0.007	a	a	a	a	a	a
FEF25-75	0.30	11.1	0.003	a	a	a	-0.55	-3.3	0.003	a	a	a	a	a	a
DLCO	0.16	4.9	0.036	a	a	a	-0.40	-2.1	0.036	a	a	a	a	a	a
DLCO / VA	0.22	3.5	0.047	0.55	2.2	0.04	-0.66	-2.6	0.015	a	a	a	a	a	a

a: Not significant, R^2: squared multiple correlation coefficient, *F: F* value, β: Partial correlation coefficients, *t: t* value of regression coefficients.

16 patients in whom interstitial pulmonary involvement was found in HRCT had HRCT scores consistent with mild parenchymal abnormalities, there was a negative correlation between HRCT score and DLCO, FVC (r=-0.364, P=0.035; r=-0.400, P=0.019, respectively).

Multiple linear regression analysis in which age and sex were considered as independent variables and KHAI was considered as dependent variable, revealed that KHAI was not affected by sex and was related with age, while KHAI was increased with age (Table 3). In order to investigate the possible association between liver pathology (KHAI) and pulmonary data (HRCT, PFT), pulmonary data were considered as dependent variables, KHAI and possible factors affecting pulmonary pathologies including age, sex and amount of cigarette smoking were considered as independent variables, then separate multiple linear regression analyses for HRCT score, VC, FVC, FEV1, FEF25-75, DLCO, DLCO/VA were performed. HRCT score, VC, FVC, FEV1, FEF25-75, DLCO, and DLCO/VA were related with age. HRCT score was positively correlated with age, whereas others decreased with increasing age (negative correlation). When multiple linear regression analysis was used, no significant relation was found between liver pathology and any of the PFT (except DLCO/VA) or HRCT score (Table 4). Although 16 patients had signs consistent with interstitial involvement in HRCT, our findings showed that this was not directly related with liver pathology.

DISCUSSION

In our study, some patients with HCV infection had a mild decrease in PFTs. However, there was no significant difference between patients and controls in PFTs. We determined that there was a significant difference between patients and controls according to the findings in thorax HRCT. The age was related with both pulmonary involvement and liver pathology.

Although the relationship between pulmonary fibrosis and HCV infection was first suggested by Ueda *et al*[9], Irving *et al*[15] held on opposite point. In a study, which investigated 300 patients with clinically evident HCV infection for the presence of pulmonary fibrosis, HRCT assessments revealed a moderate degree of pulmonary fibrosis in 4 cases and severe pulmonary fibrosis in another 4 cases. In all of these 8 cases, there were various degrees of decreases in diffusion capacity that correlated with HRCT findings and less frequently restriction in PFT parameters[5].

In our study, FEF25-75 was below 80% of the predicted value in a small group of patients (5 patients) with normal FEV1 and VC. Decrease in FEF25-75 and normal VC and FEV1 might be an indicator of an early stage small airway disease. Similar to our findings, Mimori *et al*[16] stated that in sarcoidosis patients with normal FEV1 and VC, the increase in the ratio of maximum expiratory flow rate at 50% of vital capacity (V50) to maximum expiratory flow rate at 25% of vital capacity (V25) was a finding of the early stage manifestation of small airway disease.

There are contradictory results in the literature about correlations between PFT and HRCT score in patients with pulmonary fibrosis. Also, studies generally revealed a negative correlation between PFT and HRCT score[5,13,17]. We detected a negative correlation among HRCT, FVC and DLCO in patients with HCV infection.

As HCV infections generally follow a silent course and as they are rarely diagnosed at the acute phase, onset of the disease usually could not be determined[2]. So, duration of the disease was not investigated among factors that would possibly affect the development of pulmonary involvement. HRCT score and PFT parameters were related with age. In our opinion, because duration of HCV infection may not be accurately determined, age may indirectly be a sign of the duration of the disease.

KHAI was related with DLCO/VA and age in our study. Chronic HCV infection is known to result in moderate to severe disorders in liver, particularly in elderly and alcoholics[18]. In our patients, neither any of the PFT parameters except DLCO/VA nor HRCT score was affected by KHAI (Table 3). Change in DLCO was probably related with age. Because KHAI is for the evaluation of liver parenchyma, we believe that it does not reflect the intensity of extrahepatic manifestations.

It is clearly revealed that HCV infection does not only affect

the liver, it also has many systemic manifestations. However few studies about its effect on the lung showed that there was an uncertain relationship between HCV infection and pulmonary involvement[4,18]. We found a negative correlation between HRCT score and DLCO in chronic HCV patients. In some patients (six cases), there was a decrease in DLCO despite normal HRCT findings. Many studies have shown that DLCO could decrease without presence of any radiological finding in the early stage of fibrosis and this decrease might become more significant later[19-21].

Chronic hepatitis C virus infections may cause mild pulmonary involvement without any pulmonary symptoms resulting in a minimal decrease in PFT. In our study, we found that pulmonary involvement was not related with the degree of liver pathology. The relationship between liver pathology and pulmonary involvement must be investigated in large patient population, and also, it should be detected whether alveolitis can demonstrate the early stage pulmonary involvement. However, we suggest that chronic HCV infection patients, particularly elder ones, might be carefully evaluated by HRCT and DLCO even though they have normal chest X-ray or other PFT parameters.

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• VIRAL HEPATITIS •

Expression of platelet-derived growth factor-BB in liver tissues of patients with chronic hepatitis B

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Abstract

AIM: To study the relationship between expression of platelet-derived growth factor-BB (PDGF-BB) and fibrogenesis in chronic hepatitis B.

METHODS: Hepatic tissues from 43 patients with chronic hepatitis B were embedded in paraffin. The sections were stained with HE and picric acid-sirius red to determine inflammatory activity and fibrosis stages. PDGF-BB expression was detected by immunohistochemistry and assessed semiquantatively. Levels of serum hyaluronic acid (HA), pro-collagen III (PCIII), collagen IV (IV-C) and laminin (LN) were examined by radioimmunoassay (RIA).

RESULTS: The expression level of PDGF-BB was found to be positively correlated with inflammatory activity, fibrosis stage and grade of histological findings (τ=0.58, 0.55, 0.55, P<0.01). The positive correlation was also observed between tissue level of PDGF-BB expression and contents of HA, PCIII, IV-C and LN in the circulation (r=0.52, 0.32, 0.40, 0.33, P<0.05).

CONCLUSION: PDGF-BB may play some role in the development and progression of liver fibrosis.

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INTRODUCTION

In chronic liver diseases, fibrosis is one of the parameters indicating a progressive process leading to cirrhosis. Hepatic stellate cells (HSCs) are considered to play a central role in the pathogenesis of liver fibrosis[1,2]. During the process, HSCs proliferate and differentiate into myofibroblast-like cells, synthesizing various extracellular matrix (ECM) components including collagen[2,3].

Platelet-derived growth factor (PDGF) is the most potent mitogen for HSCs[4-6], which were currently indicated as the principle cells producing connective tissue in fibrotic liver[7-9]. PDGF was currently indicated as a major inflammatory growth factor playing a central role in the repair process after acute and chronic tissue injuries. Several recent studies have demonstrated a pathogenic role of PDGF in several chronic inflammatory disorders including glomerulonephritis[10,11], scleroderma[12], rheumatoid arthritis[13], idiopathic pulmonary fibrosis[14] and atherosclerosis[15,16]. The presence of inflammatory infiltrates and the excessive deposition of collagenous matrix are the prominent features of chronic hepatitis. Along these lines, PDGF may also be involved in hepatic fibrogenesis.

PDGF consists of two polypeptide chains (A and B). Three isoforms have been described, namely PDGF-AA, -AB, -BB[17-19]. Recent studies have shown that PDGF-AB and -BB isoforms are more mitogenic for HSCs than PDGF-AA[6]. Thus, the expression of the B chain is thought to be more important in hepatic fibrogenesis than that of the A chain.

In this study we used immunohistological methods to detect PDGF-BB in human liver biopsy specimens from 43 patients with chronic hepatitis B. In addition, the levels of serum hyluranic acid (HA), pro-collagen type III (PCIII), collagen type IV (IV-C) and laminin (LN) were examined by radioimmunoassay. The relationship between expression of PDGF-BB, histologic and serum parameters were evaluated.

MATERIALS AND METHODS

Liver and serum samples

Liver tissue samples with various necroinflammatory activities and at different fibrosis stages were obtained by percutaneous liver biopsies from 43 patients with HBV-related chronic hepatitis during routine diagnostic procedures. All of the patients did not receive any anti-inflammatory or anti-fibrotic treatments, such as steroids or interferon. Informed consent was obtained from the patients for the use of their specimens in the investigation. The biopsy specimens were fixed in 10% neutral formalin and embedded in paraffin. Sections of 6 μm in thickness were used for morphological and immunohistochemical examinations. Serum samples were collected and stored at -20 ℃.

Histology

Paraffin sections were stained with hematoxylin and eosin (H&E). Alternatively, the Sirius red stain method was used to demonstrate fibrous tissue components. A polarization microscope (DMLB, Leica, Wetzlar, Germany) was used to distinguish type I from type III collagen fibers[20]. Grades of necroinflammation (0-4) and stages of fibrosis (0-4) were assessed according to the well-established criteria[21,22]. The grades and stages were scored as follows: $0=2^0$, $1=2^1$, $2=2^2$, $3=2^3$, and $4=2^4$.

Immunohistochemistry

The deparaffinized sections were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated in 3% H_2O_2/methanol for 20 minutes to block endogenous peroxidase. After washed three times in PBS, 5 min each, the sections were heated for 10 min in 0.01 M citrate buffer (pH 6.0) using a microwave oven, and then washed three times in PBS, 5 min each, and incubated with a rabbit antibody against human PDGF-BB (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA)

at a dilution of 1:50 in PBS at room temperature for 5 hours. After washed, the immunologic reaction was demonstrated using a kit (Beijing Zhongshan Biotechnology Co., Ltd. Beijing, China) and visualized in a solution containing 3, 3' -diaminobenzidine tetrahydrochloride (DAB). The slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated, air dried, and mounted. PBS was used to substitute for the primary antibody as a negative control.

Sera-assays for hepatic fibrosis

Serum hepatic fibrosis parameters including HA, C-IV, PCIII and LN, were assessed by radioimmunoassays using the kits from Shanghai Navy Medical Institute according to the manufacturer' s instructions.

Statistical analysis

Results were expressed as mean±SD. Statistical analyses were performed with the one-way ANOVA, Kendall and Spearman rank correlation. Two-tailed tests were done. $P<0.05$ was considered statistically significant.

RESULTS

Histological evaluation

The liver tissues of all patients showed various degrees of chronic inflammation and fibrosis. The fibrosis stage was 0 in 7 patients, 1 in 17, 2 in 8, 3 in 6, and 4 in 4. The stage of inflammatory activity was 1 in 13 patients, 2 in 14, 3 in 12, and 4 in 3.

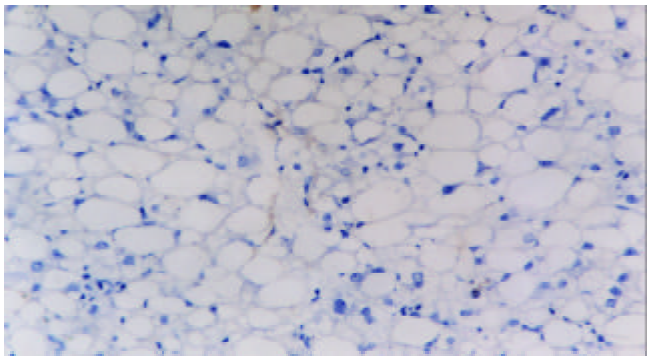

Figure 1 Immunohistochemical detection of PDGF-BB in liver biopsy of a patient with chronic hepatitis B. This case was diagnosed as stage 0. DAB, ×200.

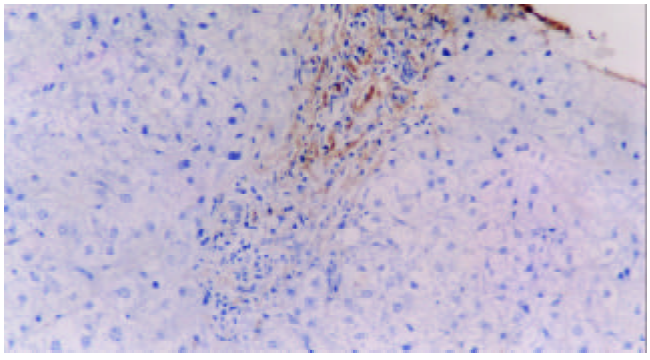

Figure 2 Immunohistochemical detection of PDGF-BB in liver biopsy of a patient with chronic hepatitis B. This case was diagnosed as stage 1. DAB, ×200.

Expression of PDGF-BB in liver biopsy samples

PDGF-BB immunoreactivity was found in a few mesenchymal cells of portal areas and fibrous septa with its products localized diffusely in the cytoplasm compartment. In intralobular areas, some perisinunoidal cells were also immunoreactive in the areas with necroinflammation. The number of positive cells increased with progression of fibrosis (Figures 1-5) and inflammation. Tables 1- 3 describe PDGF-BB expression levels of different groups respectively. Expression level of PDGF-BB was found to be positively correlated with inflammatory activity, fibrosis stages and grades of histological findings (τ=0.58, 0.55, 0.55, $P<0.01$) in Table 4.

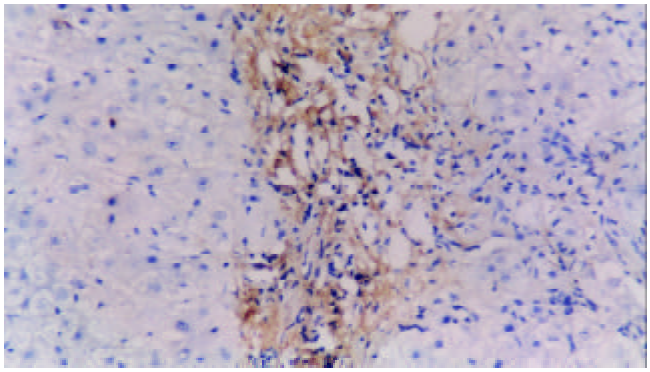

Figure 3 Immunohistochemical detection of PDGF-BB in liver biopsy of a patient with chronic hepatitis B. This case was diagnosed as stage 2. DAB, ×200.

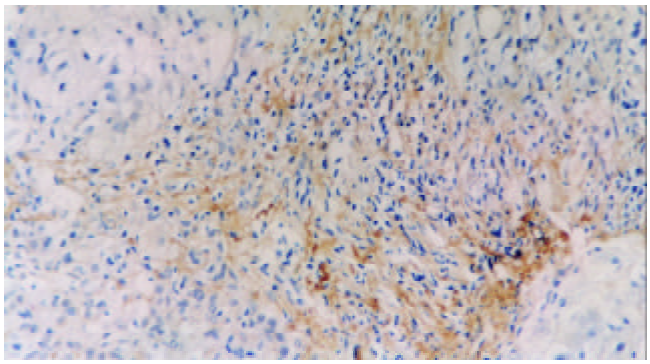

Figure 4 Immunohistochemical detection of PDGF-BB in liver biopsy of a patient with chronic hepatitis B. This case was diagnosed as stage 3. DAB, ×200.

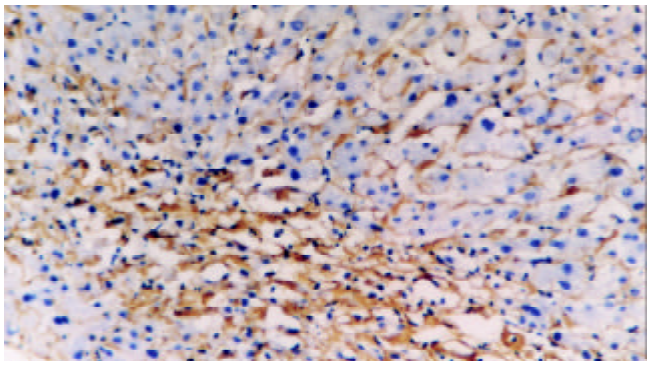

Figure 5 Immunohistochemical detection of PDGF-BB in liver biopsy of a patient with chronic hepatitis B. This case was diagnosed as stage 4. DAB, ×200.

Levels of serum hepatic fibrosis

Positive correlations were found between PDGF-BB expression levels and four serum parameters for hepatic fibrosis, including HA, LN, PC III and C-IV, and their coefficient was 0.38, 0.33, 0.32, and 0.40 ($P<0.05$), respectively (Table 5).

Table 1 PDGF-BB expression levels in liver samples with different stages of fibrosis

Stages of fibrosis	Case numbers	Expression levels (mean±SD)
S_0	7	4.71±1.50
S_1	17	6.47±2.50
S_2	8	11.63±5.66[a]
S_3	6	9.33±2.73[a]
S_4	5	16.50±5.74[a]

[a]P<0.05 *vs* S_0.

Table 2 PDGF-BB expression levels in liver tissues with different grades of necroinflammatory activity

Grades of inflammatory activity	Case numbers	Expression levels (mean±SD)
G_1	13	4.85±1.57
G_2	14	8.21±4.17[a]
G_3	12	10.67±4.12[b]
G_4	4	17.33±6.11[b]

[a]P<0.05 *vs* G_1; [b]P<0.01 *vs* G_1.

Table 3 PDGF-BB expression scores in liver tissues of different histological grading groups

Grades of histological finding	Cases numbers	Expression levels (mean±SD)
Mild	27	6.59±3.58
Moderate	10	10.20±4.16[a]
Sever	6	15.60±5.37[b]

[a]P<0.05 *vs* mild group; [b]P<0.01 *vs* mild group.

Table 4 Relationship between PDGF-BB expression levels in liver tissues of different fibrosis stages, necroinflammatory activity and histological grades

	Coefficients as compared with PDGF-BB expression levels (τ)
Stages of fibrosis	0.55[b]
Grades of inflammatory activity	0.58[b]
And histological findings	0.55[b]

[a]P<0.05; [b]P<0.01.

DISCUSSION

Liver fibrosis has been to be resulted from architectural remodeling and progressive deposition of ECM components such as proteoglycans, fibronectin, and collagen molecules[23]. It is one of the major factors affecting the clinical course of chronic liver diseases. Active fibrogenesis is frequently preceded by, and associated with inflammation. Fibrogenic growth factors and cytokines released by inflammatory cells can promote the proliferation of fat-storing cells, and designate HSCs, which were considered to be the main cellular source of matrix proteins in the liver[24,25]. In HSCs isolated from rat, mouse or human liver and activated in culture, the dimeric forms of PDGF, either PDGF-AB or -BB, appeared significantly more potent than that of -AA[26-28]. The results of these *in vitro* studies implicated that an increased expression of PDGF might occur also *in vivo* after liver tissue injury. The current study showed that most of the cells immunoreactive for PDGF-BB were located in portal areas. In intralobular areas, the positive cells were seen mainly in the areas with necroinflammation. In addition, the number of cells expressing PDGF-BB was correlated both to the inflammatory activity and to the fibrosis progression. We consider that PDGF may enhance the tissue repair after acute liver injury. In chronic liver diseases, however, the presence of reiterative tissue damage associated with a persistent inflammatory state may cause a sustained release of PDGF involved in the deposition of extracellular matrix. In this context, the prolonged effects of this growth factor on fat-storing cells may contribute to the development of tissue fibrosis rather than to effective tissue repair. Therefore, PDGF-BB may play an essential role in the development and progression of liver fibrosis. The management of PDGF activity by antagonists may prevent aggressive liver fibrosis and improve prognosis of hepatitis B.

It has been well known that collagen type I was the predominant ECM component in fibrosis and cirrhosis, but it required specific procedures to discriminate different collagen fibers on tissue sections[29,30]. We used sirius red stain to subtype the fibers as described by Zhang *et al*[20]. Under the polarization microscope, collagen types I and III were stained red and green, respectively.

Our data showed that the expression levels of PDGF-BB in the liver were correlative with the proposed serum parameters for hepatic fibrosis, including HA, LN, PC III and C-IV. ECM is a complex of macromolecules that includes collagens, proteoglycans and glycoproteins. In fibrotic liver tissue there is an increase in all of these matrix components, and they increase in serum in patients with chronic hepatitis B or liver cirrhosis. These ECM components have been used as a serum marker of hepatic fibrosis. Therefore, serum levels of connective tissue metabolites are related, to some extent, with the amount of ECM in the liver. Wang *et al*[31] reported that serum fibrosis markers were fairly well correlated with the staging of fibrosis. Regarding the correlation observed in this study between the serum parameters and the expression of PDGF-BB in liver specimens, tissue PDGF-BB levels may be correlative with the stages of hepatic fibrosis. These findings suggest that measurement of serum PDGF-BB may be useful in estimating the active hepatic fibrogenesis of chronic hepatitis B.

Table 5 Relationship between serum fibrosis parameters and PDGF-BB expression levels in liver tissues of different fibrosis stages

Staging of fibrosis(S)	Cases	PDGF-BB	HA (μg/L)	LN(μg/L)	PCIII(μg/L)	IV-C(μg/L)
S_0	7	4.71±1.50	102.67±60.38	138.23±17.69	137.80±35.57	58.71±17.40
S_1	17	6.47±2.50	168.74±159.76	129.97±30.15	143.91±51.13	64.12±20.26
S_2	8	11.63±5.66	434.86±360.58	156.39±39.06	163.74±70.56	86.04±41.94
S_3	6	9.33±2.73	430.60±325.37	172.65±39.77	192.65±40.05	115.60±30.62
S_4	5	16.50±5.74	392.30±245.53	163.25±26.26	154.68±22.24	122.10±21.34
Coefficients (r)		...	0.38[a]	0.33[a]	0.32[a]	0.40[b]

[a]P<0.05; [b]P<0.01.

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• VIRAL HEPATITIS •

Viral replication modulated by synthetic peptide derived from hepatitis B virus X protein

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Abstract

AIM: A strategy for viral vaccine design is the use of conserved peptides to overcome the problem of sequence diversity. At present it is still unclear whether conserved peptide is safe as a candidate vaccine. We reported it here for the first time not only to highlight the biohazard issue and safety importance for viral peptide vaccine, but also to explore the effect of a fully conserved peptide on HBV replication within the carboxyl terminus of HBx.

METHODS: We synthesized the fully conserved peptide (CP) with nine residues, FVLGGCRHK. HBV-producing 2.2.15 cells were treated with or without 3.5 μM CP for 36 hours. Quantitative detection of viral DNA was performed by real-time PCR. HBV antigens were determined by enzyme-linked immunoadsorbent assay (ELISA). Quantitative analyses of p53 and Bax proteins were based on immunofluorescence. Flow cytometry was performed to detect cell cycle and apoptosis.

RESULTS: Both extracellular and intracellular copies of HBV DNA per ml were significantly increased after incubation with 3.5 μM of CP. HBsAg and HBeAg in the cultured medium of CP-treatment cells were as abundant as untreated control cells. CP influenced negatively the extracellular viral gene products, and 3.5 μM CP could significantly inhibit intracellular HBsAg expression. In response to CP, intracellular HBeAg displayed an opposite pattern to that of HBsAg, and 3.5 μM CP could efficiently increase the level of intracellular HBeAg. Flow cytometric analyses exhibited no significant changes on cell cycle, apoptosis, p53 and Bax proteins in 2.2.15 cells with or without CP.

CONCLUSION: Together with the results generated from the synthetic peptide, we address that the conserved region, a domain of HBx, may be responsible for modulating HBV replication. As conserved peptides from infectious microbes are used as immunogens to elicit immune responses, their latent biological hazard for human beings should be evaluated.

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INTRODUCTION

Human hepatitis B virus (HBV) is known as an important cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma[1]. Although the incidence of new infections has decreased after the introduction of vaccination programmes, HBV infection remains an important global health problem, with the number of chronic HBV carriers exceeding 350 million worldwide[2]. HBV is a small DNA virus belonging to the hepadnavirus family that includes hepatitis viruses of woodchuck, ground squirrel, Peking duck, *etc*. These viral species all contain a highly conserved small open reading frame (ORF) encoding HBV x protein (HBx). HBx is a multifunctional viral regulator that modulates transcription, signaling pathways, protein degradation, and cell responses to genotoxic stress. These modulations affect viral replication and viral proliferation, directly or indirectly. HBx also affects cell cycle checkpoints, cell death, and carcinogenesis[3].

HBx is well conserved among the mammalian hepadnaviruses[4,5]. Regarding the viral life cycle, in related woodchuck hepatitis viruses, ablation of their X protein start codon or creation of C-terminal truncations has been found to decrease viral replication *in vitro* and to inhibit the establishment of productive infection *in vivo*[6,7]. It is not known which pathways influenced by HBx are needed or sufficient to establish an environment required for viral replication. HBx could not only form intracellular aggregates by itself [8-11], but also involve clumping and organelle aggregation leading to an abnormal mitochondrial distribution[12,13]. The precise role of HBx in the HBV life cycle remains uncertain.

Several novel HBV therapeutic peptides containing immunodominant T helper (Th) and cytotoxic T lymphocyte responses (CTL) epitopes of HBV have been screened out. Epitope-based HBV vaccines were designed and synthesized[14]. Epitope peptides derived from HBx have been used in peptide vaccines to induce specific cellular immunity[15]. In order to overcome the problem of sequence diversity, a strategy for vaccine design was the use of conserved peptides[16]. The biological importance of HBx in HBV replication remains largely undefined. In transient transfection assays the full-length X-gene encoding a product in cells appeared to form intracellular aggregates and to accumulate in large granules, with a tendency of apoptosis[9]. There was a fully conserved nine amino acid sequence within the carboxyl terminus of all HBx. Deletion of this segment resulted in a drastic loss of transactivation activity of HBx[17]. To obtain a different approach to investigate the possible functions of the X gene in HBV replication, we recently performed experiments with synthetic peptide derived from HBx. The peptide could avoid forming aggregates and be employed to investigate the effect of HBx on HBV replication. We reportet it here for the first

time to highlight the biohazard issue and safety importance regarding the synthetic peptide used as a candidate vaccine.

MATERIALS AND METHODS

In vitro synthesis of conserved peptide

HBx sequences of hepadnaviruses used for analysis were taken from GenBank. Accession numbers were as follows: arctic ground squirrel hepatitis B virus, U29144; woodchuck hepatitis virus, M19183; woolly monkey hepatitis B Virus, NC_001896; orangutan hepadnavirus, NC_002168; hepatitis B virus subtype adr, D12980; and hepatitis B virus subtype adw, M54923. Sequence alignment was performed with the software provided by Vector NTI (USA). The fully conserved peptide of nine residues from HBx, FVLGGCRHK (Figure 1), was synthesized by the solid-phase method (GL Biochem Ltd.). Purity (>97%) was assessed by high-pressure liquid chromatography, amino acid analysis, and molecular weight determination by mass spectrometry.

Arctic ground squirre hepatitis B virus	123	SRLPLFVLGGCRHKYM
Woodchuck hepatitis virus	123	PRLSIFVLGGCRHKCM
Woollymonkey hepatitis B virus	125	PRLKVFVLGGCRHKLV
Orangutan hepadnavirus	127	IRLKVFVLGGCRHKLV
Hepatitis B virus subtype adr	127	IRLKVFVLGGCRHKLV
Hepatitis B virus subtype adw	127	IRLKVFVLGGCRHKLV

Figure 1 Alignment of amino acid sequences of HBx from six mammalian hepadnaviruses.

The numbers refer to the first amino acid of each peptide. Underlined region is a fully conserved sequence.

Cell culture treated with peptide

2.2.15 cell line containing HBV ayw strain genome derived from a human hepatoblastoma HepG2 cells[18], was grown and cultured in Dulbecco' s modified Eagle' s medium (Gibco Life Technologies) supplemented with 10% heat-inactivated fetal calf serum. 2.2.15 cells were seeded into 24-well tissue culture plates. Once cell culture dishes were subconfluent (-5×10^6 cells), the culture medium was replaced by the same amount of fresh media harboring CP. Cells were incubated with 3.5 μM CP for 36 hours, the culture medium was collected for the analysis of extracellular HBV DNA and HBV antigens. The cells were washed and detached from the dishes. The collected cells were lysed. After centrifugation, the supernatant was stored for the analysis of intracellular HBV genomic forms and HBV antigens. The protein concentration was estimated by Bradford method.

Real-time PCR assay

Fluorescence quantitation of HBV DNA was performed with real-time PCR reagent kit (Shenzhen PG Biotech, China). The primers and probe were selected in the S gene of HBV genome and generated a product of 70 bp. HBV DNA was extracted from 100 μl of the sample. Real time PCR was done using 2 μl of HBV DNA, 0.06 μl of UNG (1 u/μl), 0.4 μl Taq (5 u/μl) and 37.6 μl PCR mix. After incubation for 5 min at 37 ℃, the DNA polymerase was activated at 94 ℃ for 1 min. The PCR cycling program consisted of 42 two-step cycles of 5 s at 95 ℃ and 30 s at 60 ℃. Viral DNA was extracted and purified from 100 μl of the sample. Amplification and detection were performed with a Line-gene real-time PCR analysis system (Japan). Three independent experiments were performed.

HBsAg and HBeAg assay

Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined using licensed ELISA kits (Wantai Biotech Inc., Beijing) following the manufacturer' s package insert procedure.

Flow cytometric analysis

2.2.15 cells treated with or without 3.5 μM CP for 36 hours were released by trypsinization and resuspended in phosphate-buffered saline at a density of 2 × 10^6 cells/ml. Quantitative analyses of p53 and Bax proteins were based on immunofluorescence of cells stained with fluorescein isothiocyanate-conjugated monoclonal antibodies (PharMingen, USA) respectively. Cell cycle and apoptosis were monitored using propidium iodide staining of nuclei. Flow cytometry was performed and analyzed on a Becton Dickinson flow cytometer using CellQuest software.

Statistical analysis

Statistical analysis was performed by Student' s *t* test. The data were analyzed with SigmaPlot 2000 software (SPSS Inc., USA). Differences were considered statistically significant when *P* value was less than 0.05.

RESULTS

Enhancement of HBV DNA replication after CP treatment of 2.2.15 cells

Parallel cultures, with or without CP, were maintained at 37 ℃ for 36 hours, and then investigated for the presence of viral DNA in cell lysates. HBV DNA was analyzed in a quantitative manner for the overall levels of HBV DNA (both extracellular and intracellular DNA) and the relative rate of HBV replication (intracellular DNA). HBV DNA was analyzed by real-time PCR. As shown in Figure 2, both extracellular and intracellular copies of HBV DNA per ml were significantly increased after incubation with 3.5 μM of CP. We observed that HBV DNA replication of treated 2.2.15 cells was substantially enhanced by the conserved peptide.

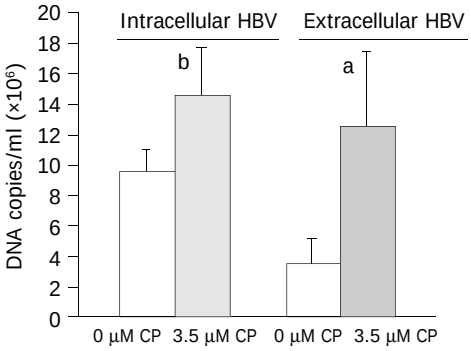

Figure 2 Quantitative detection of HBV DNA by real-time PCR. HBV DNA levels were significantly increased by 3.5 μM of CP (^aP=0.005, ^bP=0.038).

Effects of CP on expression of HBV antigens

Cell lysates and culture medium were analyzed for intracellular and extracellular viral gene products. ELISA results revealed that CP affected the expression of HBV antigens differently. As shown in Figure 3, there were no significant differences in extracellular HBsAg from 2.2.15 cells treated with or without CP, and 3.5 μM CP could significantly inhibit intracellular HBsAg expression. Changes in HBeAg were evaluated as well. It was found that there was no statistically significant difference of extracellular HBeAg when the cells were treated with CP or without CP (Figure 4). In response to CP, the intracellular HBeAg displayed an opposite pattern to that of HBsAg. We observed that CP could efficiently increase the level of intracellular HBeAg (Figure 4).

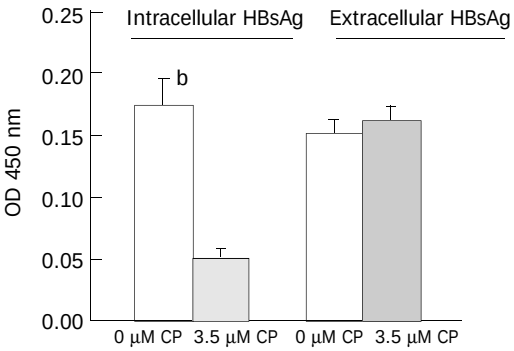

Figure 3 ELISA detection of HBsAg. Three and a half μM CP could significantly inhibit intracellular HBsAg expression ($^bP<0.001$).

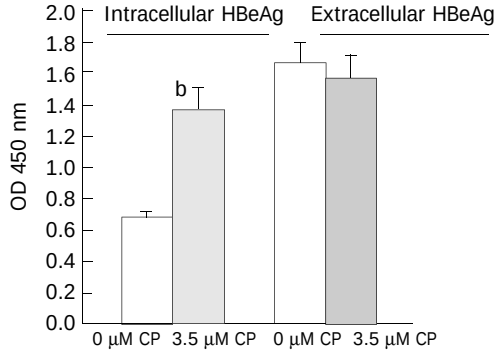

Figure 4 ELISA detection of HBeAg. The level of intracellular HBeAg was significantly increased by 3.5 μM CP ($^bP<0.001$).

Flow cytometric analysis of virus-host cells treated with CP

When fluorescein isothiocyanate-conjugated monoclonal antibodies were used to assess p53 and Bax proteins, 2.2.15 cells treated with CP or without CP displayed no significant changes (Figure 5). As shown in Table 1, the results exhibited no significant changes of cell cycle and apoptosis in 3.5 μM CP treated cells compared with the cells not treated by CP.

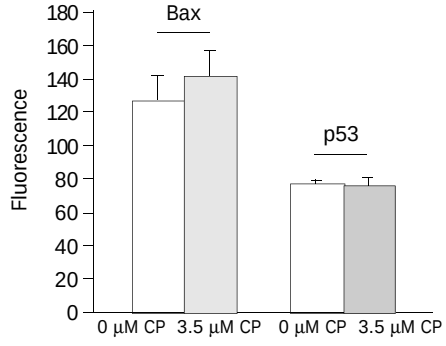

Figure 5 Flow cytometric analysis of Bax and p53 proteins.

Table 1 Percentage of apoptosis and cell cycle phase

HepG2 cells	Apoptosis	G0/G1	G2-M	S
0 μM CP	0.39±0.01	81.21±0.51	11.09±2.11	7.71±2.62
3.5 μM CP	0.62±0.27	79.87±1.22	10.24±0.99	9.89±0.30

DISCUSSION

HBV is a 3.2 Kb DNA virus with only four genes having been identified namely C, S, P, and X[19]. The C gene codes for the core protein and the e antigen, the S gene codes for three related viral envelope proteins known as surface antigens, the P gene codes for the viral DNA polymerase, and the X gene codes for a 16.5-kDa protein. HBV molecule is well organized. The X gene could overlap the viral polymerase gene and the precore gene[20,21], thus increasing the utilization of the small genome. Uchida and colleagues analyzed nucleotide (nt) sequences of HBx from more than 130 clinical HBV isolates. They attempted to establish the correlation of nt substitutions with clinical pathological characteristics. They found the X gene (465nt) was crucial for the replication and expression of HBV because HBx could transactivate HBV DNA, and contained the core promoter, enhancer II, and two direct repeats. There were several mutational hotspots, some of which seemed to relate to the immunological epitopes of the X protein. One was an 8-nt deletion between nt 1 770 and 1 777, which could truncate 20 amino acids from the carboxyl terminus of HBx. This deletion could lead to the suppression of replication of HBV DNA[22]. There was a fully conserved nine amino acid sequence within the carboxyl terminus of all HBx. We synthesized the fully conserved peptide with nine residues, FVLGGCRHK, and quested for its physiological role in regulation of HBV replication.

HBx is regarded as an important multifunctional protein for the viral life cycle and viral-host interactions. HBx has been shown to be essential for viral proliferation in the woodchuck[6,7], but was not central to HBV replication and virion export[23]. In the absence of a convenient animal model system for studying HBV replication, the transgenic mouse could provide an alternative choice. The transgenic mice HBx was not absolutely essential for HBV replication and virion secretion, but replication was significantly decreased in the absence of X protein[24]. Utilizing real time quantitative PCR, we found that CP could increase both the level of intracellular HBV DNA and the production of HBV DNA secretion.

HBsAg and HBeAg were translated from preS/S mRNA and precore mRNA individually. In this report, we demonstrated that CP could induce intracellular HBeAg expression and downregulate intracellular HBsAg expression. The results agreed closely with a recent observation of Xu *et al*., who found that the presence of HBx increased the level of C gene transcripts approximately three folds in mtTg04 mice. HBx had no apparent effect on the RNA level of the S gene in this mouse line[25]. Our result was also supported by the findings of Reifenberg' s research group[26]. They found that the X gene provided in trans could stimulate the expression of C gene when they studied transgenic mice carrying subgenomic HBV DNA. Our data suggest that HBsAg and HBeAg in cultured medium of CP-treated cells were as abundant as untreated control cells. This might be due to the basal level released from 2.2.15 cells before incubated with CP. CP had a negative influence on extracellular viral gene products. Considering the possible influence of CP on the status of host cells might alter the biologic properties of HBV, we performed analysis of cell cycle and apoptosis. Our results showed no significant cellular changes by CP. Despite the association of multiple activities with HBx, none of them appeared to provide a uniform hypothesis regarding the true biological functions of HBx[27]. Taking advantage of the synthetic peptide, we are in an attempt to identify different roles of a domain in HBx in HBV replication.

Synthetic peptide derived HBx may be responsible for modulating HBV replication. HBx has been found to be a short-lived protein and proteolysed by a ubiquitin proteasome pathway[28,29]. We think that some short peptides converted from HBx through intracellular degradation process may still remain their basic functions for HBV replication.

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• BASIC RESEARCH •

Antiangiogenic effect of somatostatin receptor subtype 2 on pancreatic cancer cell line: Inhibition of vascular endothelial growth factor and matrix metalloproteinase-2 expression *in vitro*

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Abstract

AIM: To investigate the anti-angiogenic effect of somatostatin receptor subtype 2 (SSTR2) gene transfer into pancreatic cancer cell line PC-3, and the mechanisms involved in this effect.

METHODS: The full length human SSTR2 cDNA was introduced into pancreatic cancer cell line PC-3 by lipofectamine-mediated transfection. Positive clones were screened by G418 and stable expression of SSTR2 was detected by immunohistochemistry SABC methods and RT-PCR. Enzyme-linked immunosorbent assay (ELISA) was used to detect vascular endothelial growth factor (VEGF) levels in the cell culture supernatants of SSTR2-expressing cells, vector control and mock control cells. Furthermore, the expressions of VEGF and matrix metalloproteinase-2 (MMP-2) were detected by immunohistochemistry SABC methods and RT-PCR in these cells.

RESULTS: VEGF levels in the cell culture supernatants were significantly reduced in the SSTR2-expressing cells (first week, 172.63±21.2 ng/L and after two months, 198.85±26.44 ng/L) compared with the vector control (first week, 790.39±86.52 ng/L and after two months, 795.69±72.35 ng/L) and mock control (first week, 786.42±90.62 ng/L and after two months, 805.32±84.36 ng/L) ($P<0.05$). The immunohistochemical assay showed a significant reduction of the integral optical density of VEGF and MMP-2 in the SSTR2-expressing cells (42.25±8.6 and 70.5±6.25, respectively) compared with the vector control (85.75±12.9 and 110.52±13.5, respectively) and mock control (82.6±9.28 and 113.56±9.62, respectively) ($P<0.05$). Conversely, the average gray value of VEGF and MMP-2 was significantly increased in the SSTR2-expressing cells (121.56±8.43 and 134.46±19.95, respectively) compared with the vector control (55.72±5.6 and 62.26±12.68, respectively) and mock control cells (58.48±6.2 and 65.49±9.16, respectively) ($P<0.05$). Moreover, the expressions of VEGF mRNA and MMP-2 mRNA were significantly reduced in the SSTR2-expressing cells (0.1384±0.017 and 0.2343±0.070, respectively) compared with the vector control (1.024±0.117 and 0.806±0.119, respectively) and mock control (1.085±0.105 and 0.714±0.079, respectively) ($P<0.05$).

CONCLUSION: The expression of reintroduced human SSTR2 gene exerts its antiangiogenic effects by down-regulating the expressions of the factors involved in tumor angiogenesis and metastasis, suggesting SSTR2 gene transfer as a new strategy of gene therapy for pancreatic cancer.

Kumar M, Liu ZR, Thapa L, Chang Q, Wang DY, Qin RY. Antiangiogenic effect of somatostatin receptor subtype 2 on pancreatic cancer cell line: Inhibition of vascular endothelial growth factor and matrix metalloproteinase-2 expression *in vitro*. *World J Gastroenterol* 2004; 10(3):393-399
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INTRODUCTION

The antiproliferative action of somatostatin (SS)/analogues is signaled by specific G-protein coupled receptors, and up to date, six different subtypes (SSTR-1, -2A, -2B, -3, -4, and -5) have been cloned and functionally characterized in various cell systems, including pancreas, adrenal cortex, and brain tissue[1]. However, several studies demonstrated that somatostatin receptor subtypes (SSTRs) were strongly expressed in the normal pancreas, whereas not only a desensitization or mutation of these receptors occurred in pancreatic tumors[2], but also expression of these receptors, especially SSTR2, was frequently lost in human pancreatic adenocarcinomas[3-5]. Despite remarkable biochemical properties of SS analogues *in vitro*, poor therapeutic results with them in Phase I/II clinical trials against the majority of cases of pancreatic cancers[5-7], were due to the loss of gene expression for SSTR2 in pancreatic cancers and relatively low expressions of SSTR3 and SSTR5[8].

VEGF, also known as a vascular permeability factor, is highly expressed in various types of tumors, and is an endothelial cell (EC) specific mitogen. VEGF via binding to its high affinity receptors (Flt-1/VEGFR-1, Flk-1/KDR/VEGFR-2), exerts its mitogenic effect by promoting EC proliferation and migration, thereby playing a crucial role in tumor neovascularization[9,10]. Gupta *et al* reported VEGF as a survival factor for EC, rendering these cells more radioresistant[11]. Several studies showed positive correlations between tumor cell VEGF expression, blood vessel density, tumor growth and metastasis, disease progression and poor prognosis in pancreatic carcinomas[12-14], and suppression of VEGF expression attenuated pancreatic cancer cell tumorigenicity[10], suggesting that over-expression of VEGF might be associated with the aggressive phenotype of this disease and that VEGF should be an important target for anticancer therapy.

On the other hand, matrix metalloproteinases (MMPs) were reported to enhance the degradation of extracellular matrix

(ECM), and thereby hydrolyze the important components of ECM and basement membrane such as types IV, V, VII, X collagens and fibronectin, elastin, *etc* and were closely associated with the invasiveness and metastasis of tumors[15-20]. MMP-2 was the most commonly expressed MMPs in pancreatic tumor specimens but not in normal pancreas and was correlated with the aggressive phenotype (invasive and metastatic potential) of pancreatic carcinomas[21-24]. Ellenrieder *et al* demonstrated that increased expression and activation levels of MMP-2 were strongly associated with elevated expression levels of its activators MT1-MMP (Membrane type 1-MMP) and MT2-MMP (Membrane type 2-MMP) and that it played a significant role in pancreatic tumor cell invasion[25]. The selective MMP-2 and MMP-9 inhibitor MMI-166 exerted its antitumor effects on pancreatic cancer by inhibiting its invasion and angiogenesis[26].

In the present study, we evaluated the potency of introduction of exogenous SSTR2 gene as negative regulators of VEGF and MMP-2 production in pancreatic cancer cell line PC-3 for the following reasons: (1) SSTR2 was strongly expressed in normal pancreas whereas it was frequently lost in human pancreatic adenocarcinoma[2-4]. (2) Antitumor effects of SSTR2 occurred as a consequence of an SSTR2-dependent negative feedback autocrine loop, whereby SSTR2 induced the expression of its own ligand, SS, which also constitutively activated SSTR2[4,27]. (3) SS/analogues exhibited antisecretory effects by the inhibition of release of growth factors and trophic hormones, *e.g.*, growth hormone, insulin-like growth factor-1, insulin, gastrin[2]. In addition, since pancreatic cancer is characterized by the over-expression of several angiogenic factors such as VEGF, bFGF, MMP-2, *etc*, it is necessary to elucidate the effects of the genes or molecules with antiangiogenic properties on pancreatic cancer. Previous studies have reported the antiangiogenic properties of SS/analogues and SSTRs in various tumors[28,29], however the mechanisms involved in the antiangiogenic effects of SSTR2 have been poorly elucidated in this pancreatic cancer cell line PC-3 as yet. Hence, our present study demonstrated that the reexpression of SSTR2 gene in the pancreatic cancer cell line PC-3 devoid of SSTR2, exhibited its antiangiogenic effects by down-regulating the expression of angiogenic factors *in vitro* both at protein and mRNA levels.

MATERIALS AND METHODS

Materials

PC-3, a human pancreatic cancer cell line, was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Dulbecco' s modified eagles medium (DMEM), fetal bovine serum (FBS), lipofectamine and geneticin (G418) were purchaesd from Gibco BRL. The full-lenth cDNA of human SSTR2 was kindly provided by G. I. Bell (Howard Hughes Medical Institute, Chicago, IL). Monoclonal antibody of SSTR2, mouse anti-VEGF polyclonal antibody, mouse anti-MMP-2 monoclonal antibody, SABC kit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TRIZOL®reagent, RNasin, oligo(dt) 15, dNTPs, M-MLV reverse transcriptase, Taq DNA polymerase and VEGF ELISA kit were from Promega. Eukaryotic expression vector pcDNA3.1 was purchased from Invitrogen (Invitrogen, San Diego, CA).

Plasmid construction and gene transfer

Human SSTR2 cDNA was digested with *EcoRI*/*XbaI* and cloned in the *EcoRI*/*XbaI* site of pcDNA3.1. The sequence encoding the signal peptide from alkaline phosphatase was inserted into the *HindIII*/*EcoRI* site upstream of SSTR2 and an epitope tag derived from influenza virus hemagglutinin A (HA) was fused in the *EcoRI* site between the signal peptide and SSTR2. Recombinant plasmid was purified by QIA prep spin miniprep kit (QIAGEN Co).

PC-3 cells were routinely cultured in DMEM media supplemented with 10% heat-inactivated FBS, 100 u/ml penicillin and 100 u/ml streptomycin, and incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2 in air. Gene transfer was performed according to the manufacture' s protocol of lipofectamine (Gibco BRL). Briefly, about 3×10^5 cells per well containing 2 ml appropriate complete growth medium were seeded in a six-well culture plate, and incubated at 37 ℃ in a CO_2 incubator until the cells were 70% to 80% confluent. A cover slip was plated in each well before seeding. After the cells were ringed with serum free and antibiotics-free medium, the cells were transfected separately with pcDNA3.1-SSTR2 1 μg/lipofectamine 3 μL (experimental group), pcDNA3.1 1 μg/lipofectamine 3 μL (vector control), and only lipofectamine 3 μL (mock control), followed by incubation at 37 ℃ in a CO_2 incubator for 6 hours. Then the medium was replaced by DMEM culture medium containing 20% FBS. After 48 hours, two wells in each group were taken out to detect the transient expression of SSTR2 by immunohistochemical SABC methods, whereas others were continuously cultured for stable expression of SSTR2. G418 (500 mg/L) was added to select the resistant clones after 48 hours. Six days later, when most of the cells died, the concentration of G418 was decreased to 300 mg/L and cells were cultured with G418 for another 6 days. Then the medium was changed every 3 or 4 days and colonies were collected approximately 2 weeks later for the examination of stable expression of SSTR2 by immunohistochemical SABC methods and RT-PCR assay.

Confirmation of SSTR2 protein expression by immunohistochemical staining

The stable expression of SSTR2 in the experimental group cells was detected by using immunohistochemical SABC methods. All the cover slips were dried at room temperature and washed twice with PBS solution (pH 7.2), followed by the treatment with 3% H_2O_2 for 10 min at room temperature. Then the cover slips were incubated in 5% bovine serum albumin in PBS solution for 20 min to block the nonspecific antibody-binding. The cover slips were then incubated with mouse anti-human SSTR2 antibody, diluted in 1:50 in 0.5% bovine serum albumin in PBS for 12 hours at 4 ℃. The bridging antibody (biotinylated goat anti-mouse IgG) and SABC complex were diluted to 1:100 and incubated with the specimens for 20 min at 30 ℃. Finally diaminobenzidine tetrachloride (DAB) was used for color development and the cover slips were counterstained with hematoxylin. Positive rate (brown color cells) was automatically measured with the biological image analysis system 2000 (Opton Germany).

Confirmation of SSTR2 mRNA expression by RT-PCR

Total RNA was extracted separately from PC-3 cells of each group with TRIZOL®reagent following the manufacturer' s instructions. A 2 μg (treated in 5 μL DEPC water in an Ep tube) sample of total RNA was denaturalized by incubating at 70 ℃ for 5 min, and the tube was placed to ice for 3 min, and then reverse-transcribed into complementary DNA (cDNA) by using Moloney murine leukemia virus reverse transcriptase (M-MLV). Briefly, the denaturalized RNA (5 μL) was incubated for 60 min at 37 ℃ and for 5 min at 95 ℃ with 4 μL 5×reverse transcriptase buffer, 1 μL oligo (dt) 15, 1 μL RNasin (50 u/μL), 1 μL dNTPs (10 mmol/L), 1 μL reverse transcriptase (200 u/μL), and 7 μL DEPC water in a total volume of 20 μL. For polymerase chain reaction (PCR), 5 μL of the resulting cDNA, 31 μL of tripled-distilled H_2O, 5 μL of 10×PCR buffers, 3 μL of $MgCl_2$ (25 mmol/L), 1 μL of dNTPs, 1 μL of each of sense and antisense primers (10 pmol/L), 1 μL of each of sense

and antisense β-actin, and 1 μL Taq DNA polymerase (3 u/μL) in a total volume of 50 μL were added. The samples were amplified through 35 cycles, each amplification consisting of denaturation at 94 ℃ for 40 s, primer annealing at 55 ℃ for 40 s and extension at 72 ℃ for 1 min. Cycles were preceded by incubation at 94 ℃ for 5 min to ensure full denaturation of the target gene, followed by an extra incubation at 72 ℃ for 10 min to ensure full extentsion of the products. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide. The sequences of the primers for SSTR2 were sense 5' -CCCCAGCCCTTAAAGGCATGT-3' and antisense 5' -GGTCTCCATTGAGGAGGGTCC-3' (234 bp) and for β-actin, sense 5' -GTGCGTGACATTAAGGAG-3' and antisense 5' -CTAAGTCATAGTCCGCCT-3' (520 bp).

Detection of VEGF mRNA and MMP-2 mRNA expression by RT-PCR

Total RNA was extracted separately from PC-3 cells of each group with TRIZOL®reagent following the manufacturer' s instructions. RT-PCR was carried out as described above except some changes in conditions of amplification cycles. For mRNA expression of VEGF, samples of each group were subjected to PCR at an annealing temperature from 60 ℃ to 50 ℃ decreased by 0.5 ℃ per cycle for 20 cycles, followed by an additional 15 cycles at an annealing temperature of 50 ℃ for 35 s. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide and quantified by a complete gel documentation and analysis system. VEGF mRNA expression level was determined by the ratio of VEGF/β-actin protein. The sequences of primers for VEGF were sense 5' -TTGCTGCTCTACCTCCAC-3' and antisense 5' -CTCCAGGCCCTCGTCATT-3' (240 bp) and for β-actin, sense 5' -GTGCGTGACATTAAGGAG-3' and antisense 5' -CTAAGTCATAGTCCGCCT-3' (520 bp).

For mRNA expression of MMP-2, samples of each group were subjected to PCR for 33 cycles, each cycle consisting of denaturation at 94 ℃ for 1 min, primer annealing at 55 ℃ for 35 s and extentsion at 72 ℃ for 1 min. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide and quantified by a complete gel documentation and analysis system. MMP-2 mRNA expression level was determined by the ratio of MMP-2/β-actin protein. The sequences of primers for MMP-2 were sense 5' -GCGGATCCAGCGCCCAGAGAGACAC-3' and antisense 5' -TTAAGCTTCCACTCCGGGCAGGATT-3' (473 bp) and for β-actin were sense 5' -CCTTCCTGGGCAT-GGAGTCCTG-3' and antisense 5' -GGAGCAATGATCTTG-ATCTTC-3' (205 bp).

Determination of VEGF concentration in cultured supernatants by ELISA

After successful stable transfection, VEGF protein levels in the supernatants secreted by the cultured human pancreatic cancer cells of experimental group, vector control and mock control were quantitated by ELISA. To generate the conditioned medium, the cells of each group (4×10^5/well) were incubated in 1.5 ml DMEM for 48 hours. The conditioned medium was then collected and centrifuged at 12 000 rpm at 4 ℃ for 15 min, and then ELISA analysis was performed according to the manufacturer' s instructions. Furthermore, to observe the long-term antisecretory effect of SSTR2, ELISA analysis was performed two months after stable transfection. The value of OD (A_{450} values) of each well was measured at 450 nm. The supernatants were harvested in triplicate and the experiment was performed twice.

Detection of VEGF and MMP-2 expressions in vitro by immunohistochemistry

Two cover slips were plated in each well of six-well culture plates, and then the cells of each group (10^5 cells) containing 2 ml appropriate complete growth medium were seeded in each well, and incubated at 37 ℃ in a CO_2 incubator for 48 hours. All the cover slips were dried and washed three times with PBS (pH 7.2) at room temperature, followed by the treatment with the 1:1 mixture of 100% acetone and formaldehyde for 10 min at room temperatue. The expressions of VEGF and MMP-2 were detected by using immunohistochemical SABC methods as described above by using mouse anti-human VEGF polyclonal antibody and MMP-2 monoclonal antibody. Under the light microscope, positive staining (brown yellow) was located in cytoplasm and membrane for VEGF and MMP-2. For the image analysis, 150 cells of clear outline from 10 microscopic fields (15 cells in each field) were selected randomly from each group under 10×40 magnification, and the average gray value and integral optical density of each group were automatically measured by using HPIAS-1000, high resolution pathological image analysis.

Statistical analysis

Results were expressed as mean±SD and the mean values were compared by using the ANOVA (SNK, Student-Newman-Keuls test) in the SAS 8.1 software and $P<0.05$ was considered statistically significant.

RESULTS

Reexpression of SSTR2 after transfection

After 48 h *in vitro* transfection, most of the cells in experimental group demonstrated positive staining for SSTR2 as detected by immunohistochemical SABC methods, whereas almost no positive SSTR2 stainings were detected in vector control and mock control. The cells in experimental group were continuously cultured by adding G418 (500 mg/L) with 20% FBS, and then we were able to select a population of PC-3 cells resistant to the toxic effects of G418. After 2 weeks, all of these cells demonstrated positive staining for SSTR2 detected by immunohistochemical SABC methods (Figure 1). Furthermore, RT-PCR analysis of total RNA extracted from the cells of each group showed SSTR2 mRNA expression in experimental group, but not in vector control and mock control (Figure 2). The results suggested that the exogenous SSTR2 gene was successfully reexpressed in PC-3 cell line devoid of SSTR2.

Effect of SSTR2 on VEGF production in cultured supernatants

In the first week after stable transfection, VEGF levels in the cultured supernatants were significantly decreased in the cells of experimental group (172.63±21.2 ng/L) compared with those of vector control (790.39±86.52 ng/L) and mock control (786.42±90.62 ng/L) ($P<0.05$, Figure 3). Similarly, two months after stable transfection, there was still a significant inhibition of VEGF production in the cells of experimental group (198.85±26.44 ng/L) compared with those of vector control (795.69±72.35 ng/L) and mock control (805.32±84.36 ng/L) ($P<0.05$, Figure 3), however the inhibition of VEGF production in the cells of experimental group was slightly less than that in the first week (Figure 3). There were no statistical difference in VEGF levels between the vector control and mock control (Figure 3). Thus, SSTR2 could suppress the production of VEGF secreted by the cells of experimental group.

Effect of SSTR2 on VEGF mRNA and MMP-2 mRNA expression in vitro

RT-PCR analysis showed that the expression of VEGF mRNA was significantly decreased in the experimental group (0.1384±0.017) compared with the vector control (1.024±0.11)

and mock control (1.085±0.105) (P<0.05, Figures 4 and 6). Similarly, the expression of MMP-2 mRNA was also significantly reduced in the experimental group (0.2343±0.07) compared with the vector control (0.806±0.119) and mock control (0.714±0.079) (P<0.05, Figures 5 and 6). But there was no statistical difference either in VEGF mRNA or in MMP-2 mRNA expression between the vector control and mock control. These results suggested that the reexpression of SSTR2 gene could suppress the expression of VEGF and MMP-2 at mRNA level *in* vitro.

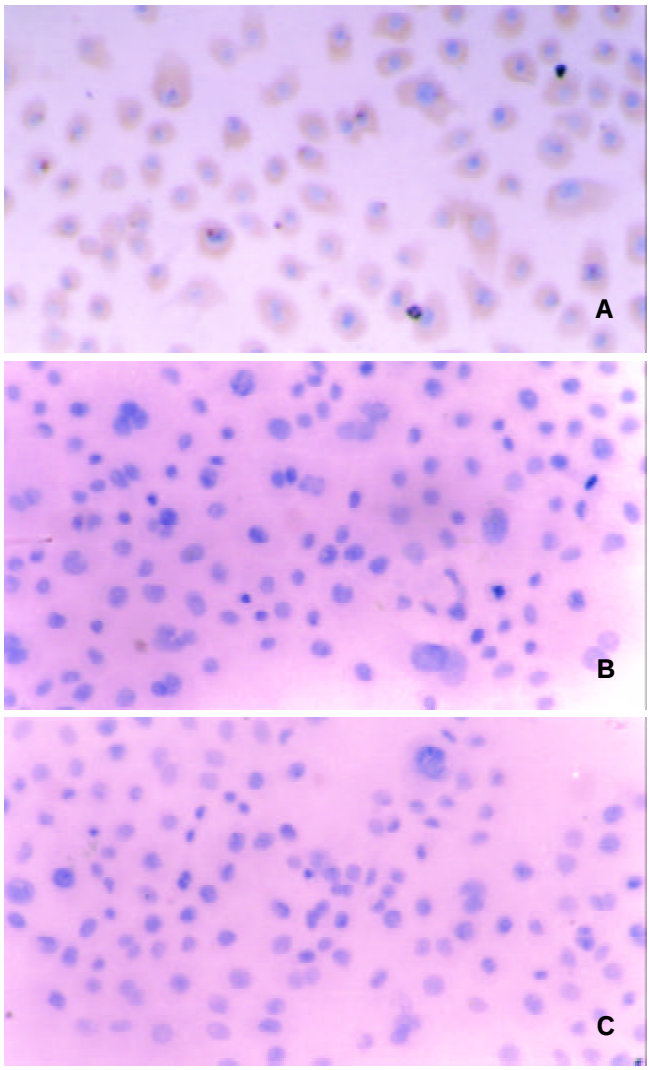

Figure 1 Immunohistochemical staining of SSTR2 expression after stable transfection The cytoplasmic brown yellow staining represents SSTR2 expression. (A) experimental group, (B) vector control, and (C) mock control (SABC×150).

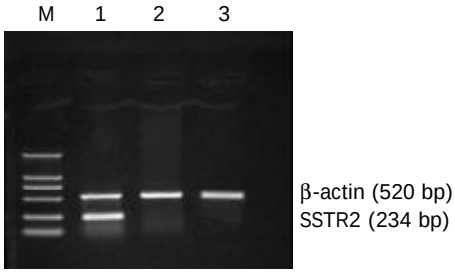

Figure 2 Expression of SSTR2 mRNA by RT-PCR analysis in experimental group, but not in vector control and mock control. Lane M: DNA marker DL 2000, Lane 1: experimental group, Lane 2: vector control, Lane 3: mock control.

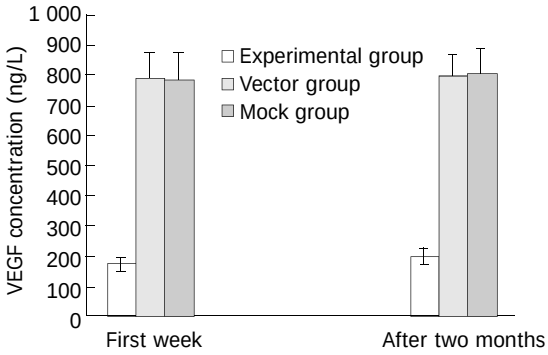

Figure 3 Inhibition of VEGF production in cultured supernatants by SSTR2. VEGF was determined in cultured supernatants by ELISA in the first week and two months after stable transfection. VEGF secretion was significantly reduced in the experimental group compared with the vector and mock controls (P<0.05). Values were expressed as mean±SD.

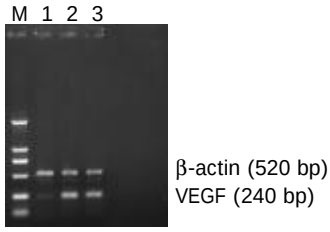

Figure 4 Weak expression of VEGF mRNA in experimental group but strong expression in vector and mock control shown by RT-PCR analysis. The expected length of PCR products was 240 bp (VEGF) and 520 bp (β-actin). Lane M: DNA marker DL 2000, Lane 1: experimental group, Lane 2: vector control, Lane 3: mock control.

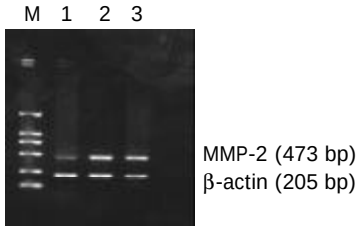

Figure 5 Weak expression of MMP-2 mRNA in experimental group but strong expression in vector and mock control shown by RT-PCR. The expected length of PCR products was 473 bp (MMP-2) and 205 bp (β-actin). Lane M: DNA marker DL 2000, Lane 1: experimental group, Lane 2: vector control, Lane 3: mock control.

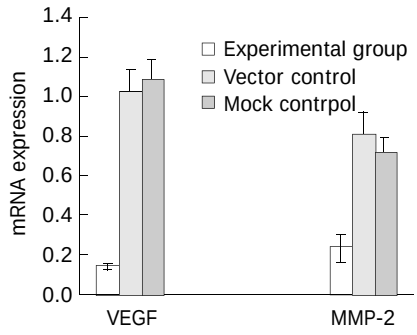

Figure 6 Significant inhibition of VEGF mRNA and MMP-2 mRNA in experimental group compared with vector control and mock control shown by quantification analysis of PCR products of VEGF and MMP-2 mRNA on 1.5% agarose gel containing ethidium bromide (P<0.05). Values were expressed as mean±SD.

Down-regulation of VEGF and MMP-2 protein expression by SSTR2

Positive staining of VEGF (Figure 7) and MMP-2 (Figure 9) was located in the cytoplasm and membrane of the cells. The immunohistochemical staining showed a significant decrease in the expression of both VEGF and MMP-2 protein in the experimental group compared with the vector control and mock control ($P<0.05$). According to HPIAS-1000 and statistical analysis, the integral optical density of VEGF staining was significantly reduced in the experimental group (42.25±8.6) compared with the vector control (85.75±12.9) and mock control (82.6±9.28) and the average gray value of VEGF staining was significantly increased in the experimental group (121.56±8.43) compared with the vector control (55.72±5.6) and mock control (58.48±6.2) ($P<0.05$, Figure 8). However, no significant difference was observed between the vector control and mock control.

Similarly, according to HPIAS-1000 and statistical analysis, the integral optical density of MMP-2 staining was significantly reduced in the experimental group (70.5±6.25) compared with the vector control (110.52±13.5) and mock control (113.56±9.62) and the average gray value of MMP-2 staining was significantly increased in the experimental group (134.46±19.95) compared with the vector control (62.26±12.68) and mock control (65.49±9.16) ($P<0.05$, Figure 10). However, no significant difference was observed between the vector control and mock control.

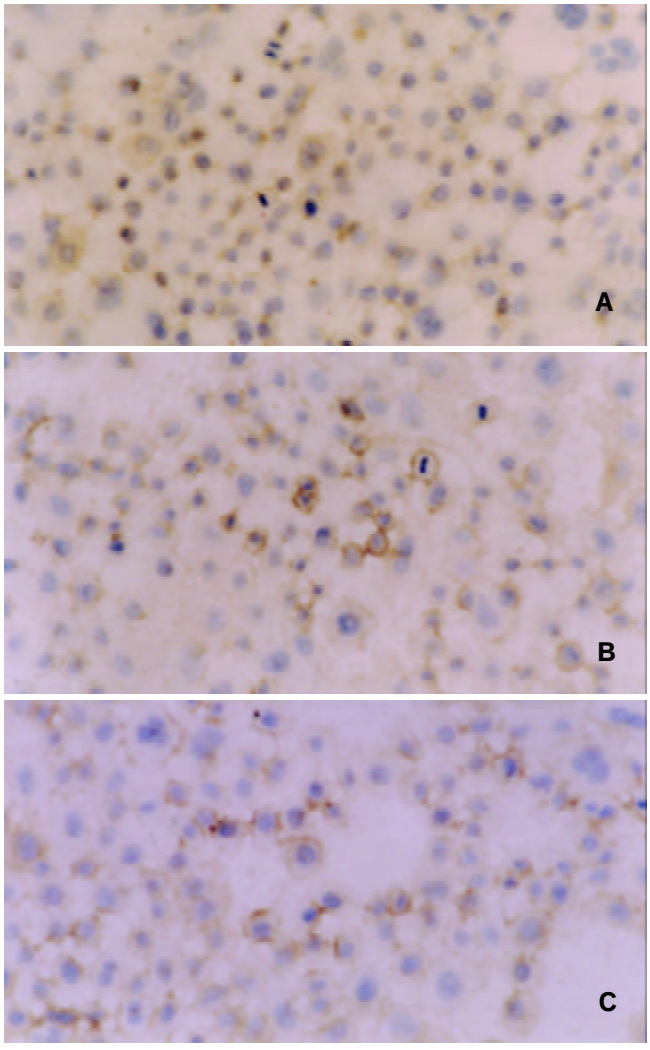

Figure 7 Location of immunohistochemical staining of expression of VEGF in cytoplasm and membrane. A: experimental group, B: vector control, C: mock control.

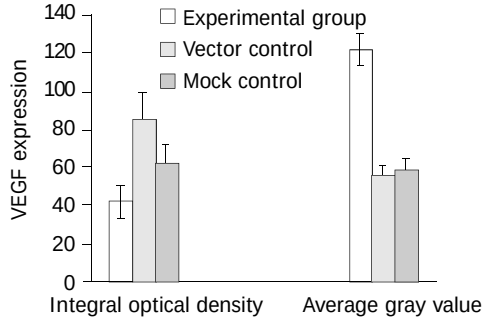

Figure 8 Down-regulation of VEGF protein expression by SSTR2. The expression of VEGF was significantly decreased in experimental group compared with vector control and mock control ($P<0.05$). Values were expressed as mean±SD.

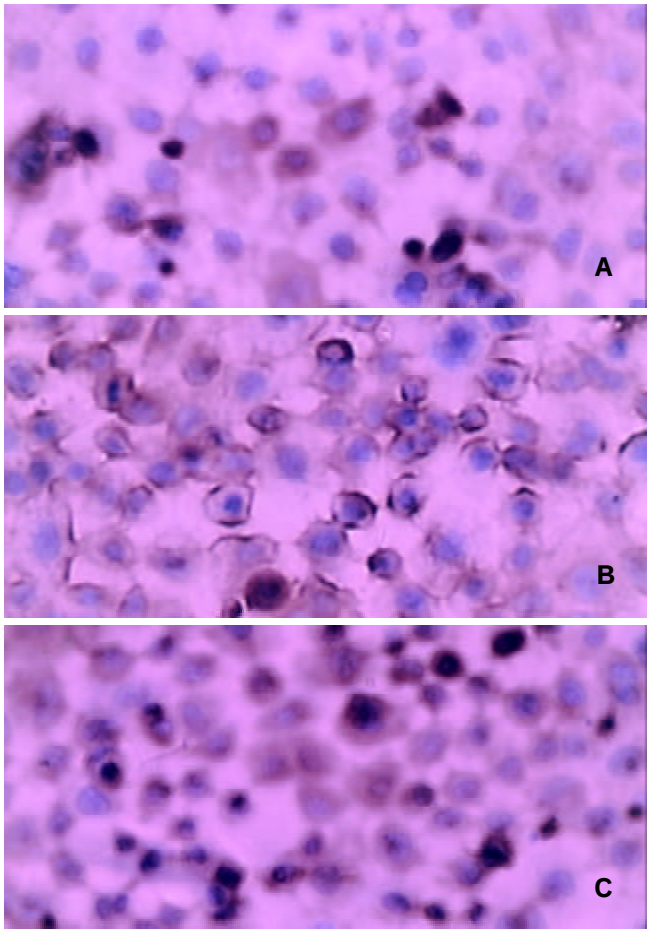

Figure 9 Location of immunohistochemical staining of expression of MMP-2 in cytoplasm and membrane. A: experimental group, B: vector control, C: mock control (SABC×400).

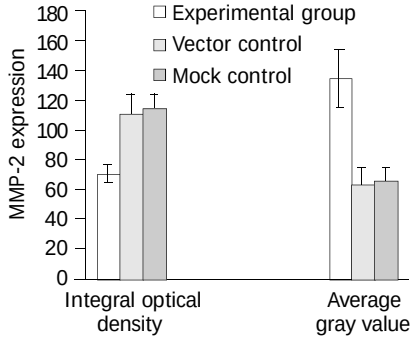

Figure 10 Down-regulation of MMP-2 protein expression by SSTR2. The expression of MMP-2 was significantly decreased

in experimental group compared with vector control and mock control (P<0.05). Values were expressed as mean±SD.

DISCUSSION

The properties of tumor cells to release and induce several angiogenic and antiangiogenic factors which play a crucial role in regulating EC proliferation, migration, apoptosis or survival, cell-cell and cell-matrix adhesion through different intracellular signalings, have been thought to be the essential mechanisms during tumor-induced angiogenesis[9]. The important steps during tumor angiogenesis, such as degradation of basement membrane by proteases and proliferation and migration of EC, were associated with the over-expression of angiogenic factors[9]. Since fundamental requirements of tumor growth are dependent on the blood supply, the antiangiogenic therapy of cancer represents a highly effective strategy for destroying tumors. In addition, antiangiogenic agents, if administered before a tumor develops or becomes vascular supply-dependent, would therefore theoretically act similarly to a vaccine in preventing tumor development, not just tumor growth. Similarly, transfer of antioncogene or molecules with antioncogenic properties constitutes one of the new therapeutic approaches to cancer. SSTR2 could act as an antioncogene in human pancreatic cancer cells and its antiproliferative and antimetastatic effects could occur as a consequence of an SSTR2-dependent negative feedback autocrine loop[4,27]. Furthermore, the loss of SSTR2 during pancreatic tumorigenesis[3-5] was found to be responsible for the poor therapeutic efficacy[5-7] and the aggressive behaviours of pancreatic carcinoma. However, to our knowledge, whether the abberent expression of SSTR2 is associated with pancreatic tumor angiogenesis, has not been reported as yet. But several previous investigations have shown that SS/analogues as well as SSTRs exhibited their antitumor effects through different pathways such as inhibition of release of growth factors and trophic hormones, *e.g.*, growth hormone, insulin-like growth factor-1, insulin, gastrin, *etc*[2]. In CAM model, the study showed that unlabeled SS analogues inhibited angiogenesis which was proportional to the ability of the analogues to inhibit growth hormone production[8]. Moreover, Mentlein *et al* reported that VEGF produced by cultured glioma cell lines constantly over-expressing SSTRs, especially SSTR2, was reduced to 25% to 80% by co-incubation with SS or SSTR2-selective agonists (octreotide and L-054 522) in a dose-dependent manner[30]. Interestingly, transfer of SSTR2 gene was found to restore the responsiveness of SSTR2-negative cells to SS analogues, and inhibited the tumorigenicity of pancreatic tumor cells *in vitro* without administration of exogenous SSTR2 ligands[4]. Similar to these, in our study, we suceesfully reexpressed exogenous human SSTR2 gene in pancreatic cancer cell line PC-3 by lipofectamine-mediated stable transfection and investigated its antiangiogenic mechanisms in the absence of SSTR2 ligand, SS.

The most commonly found angiogenic growth factors such as VEGF could contribute to the progression of many solid tumors by promoting the "angiogenic switch"[31]. In patients with pancreatic cancer, hypervascularity which was correlated with the over-expression of several angiogenic factors such as VEGF, PD-ECGF, MMP-2, *etc*, was significantly associated with tumor extension, lymph node status and shorter median survival time[9,14,32]. And also since VEGF via binding to its high affinity receptors (VEGFR) on EC, exerted its mitogenic effect by promoting EC proliferation and migration, resulting in tumor neovascularization[9,10], the VEGF-VEGFR system has been considered as a promising target for the development of antiangiogenic tumor therapy[33-37]. In our study, we observed a significant decrease in VEGF production levels in the cultured supernatants of the experimental group (cells reexpressed SSTR2) when compared with the vector control and mock control (P<0.05). We further observed that the antisecretory effect of SSTR2 was similar to or even more durable than that of SS/analogues which was time- and dose-dependent[29], because in our experiment, we observed a significant inhibition of VEGF production in the cultured supernatants of the experimental group, which persisted for at least two months after stable transfection and the inhibitory rate of SSTR2 was slightly less after two months than in the first week after stable transfection, suggesting that there was a long-term induction of SS (ligand of SSTR2) by the SSTR2-dependent negative feedback autocrine loop. By contrast, Hipkin *et al* reported that the inhibitory effects of SSTR2 gene were not permanent and stable because of its down-regulation or desensitization by long-term exposure to SS[38]. In addition, we observed a significant inhibitory effect of SSTR2 on VEGF expression both at protein and mRNA levels *in vitro* detected by immunohistochemical and RT-PCR assay, respectively. All of these antisecretory events evoked by SSTR2 expression in PC-3 cell may explain the antiangiogenic effect observed *in vitro*.

On the other hand, up-regulation of MMPs activity favoured proteolytic degradation of the basement membrane and ECM, thereby releasing angiogenic mitogens stored within the matrix, and has been linked to tumor growth and metastasis, as well as tumor-associated angiogenesis[9]. Furthermore, the aggressive phenotype of pancreatic carcinoma has been reported to be associated with over-expression of MMP-2[21-24]. Due to the high level expression of MMP-2 in clinical and experimental models of pancreatic cancer, inhibition of MMP-2 has shown a great promise with synthetic inhibitors as antitumor agents (antiangiogenesis, antiproliferative and antimetastasis) in preclinical models[39]. Here, we observed that MMP-2 was significantly down-regulated both at protein and mRNA levels *in vitro* in the experimental group when compared with the vector control and mock control, suggesting that the reexpression of SSTR2 in pancreatic cancer cell line PC-3 could decrease the aggressive phenotype of pancreatic carcinoma. Similarly, Wang *et al* reported that administration of SS analogue, octreotide, was found to inhibit the migration and invasion of gastric cancer cells *in vitro* and the metastasis of cancer *in vivo* via down-regulation of MMP-2 and VEGF expressions, thereby decreasing tumor angiogenesis[29].

In conlusion, our present study shows that the reexpression of SSTR2 gene can inhibit angiogenesis in pancreatic carcinoma by decreasing endogenous levels of VEGF and MMP-2, suggesting that restoration of SSTR2 in pancreatic cancer cells may offer an avenue for antiangiogenic therapy.

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• BASIC RESEARCH •

Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer

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Abstract

AIM: To investigate the *in vitro* effects of suicide gene therapy system of herpes simplex virus thymidine kinase gene (HSV-TK) in combination with the treatment of nucleotide analog-ganciclovir (GCV) on human pancreatic cancer, and to provide a novel clinical therapeutic method for human pancreatic cancer.

METHODS: We used a replication defective recombinant retrovirus vector GINaTK (bearing HSV-TK gene) to make packaging cell PA317 produce progeny virions. We then transferred the HSV-TK gene to target cells SW1990 using these progeny virions, and treated these gene-modified tumor cells with GCV to study the sensitivity of the cells to GCV and their bystander effects by routine MTT-method.

RESULTS: Packaging cell PA317/TK was successfully constructed, and we acquired SW1990/TK through virus progeny infection. These gene-modified pancreatic cancer cells were sensitive to the treatment of GCV compared with unmodified tumor cells (t=4.15, n=10, P<0.0025). We also observed a remarkable bystander effect by mixing two kinds of cells at different ratio.

CONCLUSION: Our data demonstrate that HSV-TK/GCV suicide gene therapy system is effective for treating experimental human pancreatic cancer, which is largely resistant to the common therapies, so the suicide gene therapy system may be a potential treatment approach for pancreatic cancer.

Wang J, Lu XX, Chen DZ, Li SF, Zhang LS. Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer. *World J Gastroenterol* 2004; 10(3):400-403

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INTRODUCTION

Pancreatic cancer is an aggressive malignancy with less than 5% of the patients alive at 5 years and 92% of the patients dead at 2 years[1,2]. Despite of the development in the three routine therapeutic methods of surgery[3], chemotherapy, and radiotherapy, the cure rate for pancreatic cancer has improved only minimally, and the overall survival of patients remains dismal. Its prognosis is extremely poor with current modes of treatment[4,5]. Therefore, it is urgent to develop effective approaches to this lethal disease. With great progresses of gene therapy in recent years[6], much of interest and effort have been focused on the treatment of pancreatic cancer. The use of pro-drug-activating genes is a promising approach for cancer gene therapy, especially herpes simplex virus thymidine kinase gene (HSV-TK) in combination with ganciclovir (GCV), which is currently used in gene therapy-based experimental trials for cancer treatment, and in clinical treatment of brain tumors[7,8].

Virus-originated HSV-TK gene is different from that of mammals, its product thymidine kinase is able to metabolize the nontoxic prodrug, GCV, into a monophosphate derivative, then phosphorylate it further into GCV triphosphate. This metabolite is incorporated into replicating DNA strands and acts as both a DNA synthesis inhibitor and a cell cycle blocker, finally leading to cell apoptosis and death[9,10], which is also called "suicide gene". The therapeutic effect of this system is also based on a "bystander effect" whereby HSV-TK gene modified tumor cells are toxic to nearby unmodified tumor cells when exposed to the antiviral drug GCV. In this study, we wanted to see whether suicide gene therapy system was effective for the treatment of human pancreatic cancer.

MATERIALS AND METHODS

Cell culture

Murine fibroblasts NIH3T3 cells and packaging cell PA317 were propagated in DMEM (Gibico) with low and high concentrations of glucose separately supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human pancreatic cancer cell line SW1990 was maintained in RPMI1640 (Gibico) supplemented with 20% FBS. Transgenic cell PA317/TK and SW1990/TK were maintained in DMEM (10%FBS) and RPMI1640 (20% FBS) respectively, both with 300 μg/ml of G418 (Promega).

Construction of package cell PA317/TK

HSV-TK gene was inserted downstream of the cytomegalovirus (CMV) promoter in a GINa plasmid containing neo open reading frame (NeoORF), the neomycin resistance gene, which is resistant to neomycin analogue G418. Packaging cells PA317 were transfected with a GINaTK plasmid vector (a gift from the Genetic Research Center of Fudan University, Shanghai, China) using Lipofectin as recommended by the manufacturer (Gibico). Transfected cells were selected with 300 μg/ml of G418 in DMEM supplemented with 10% FBS for weeks. Single-clones were selected, expanded, and maintained in G418-containing medium until further experiments. Total cell DNA were extracted for PCR using specific primer sets for HSV-TK gene (Prime 1: 609 bp-630 bp: 5' -CTACACCACACAAC ACCGCCTC-3' ; Prime 2: 1 012 bp-991 bp: 5' -TCGCAGCCAGCATAGC CAGGTC-3'). We also used scanning-electronmicroscope (SEM) to confirm the success of gene transfer and the production of viral progeny. Virus titers were determined on NIH3T3 cells by plague assay.

Transfer of HSV-TK gene to pancreatic cancer cells

One day before transfection, we seeded cells to be infected with a density that would allow them to grow logarithmically for at least 2-3 days. The transfection protocol was that the culture supernatant of PA317/TK containing progeny virion was collected and then replaced by fresh DMEM (10% FBS, without G418) within 24 hours before transfection, and passed through a 0.22 μm filter. Then the cell growth medium of SW1990 was replaced with 5 ml of viral supernatant containing 8 μg/ml polybrene to help the adhesion of viruses. Cells were returned to the incubator and incubated for 2 hours, and then 5 ml of growth medium was added and incubated overnight. The next day the supernatant was removed and the cells were fed with. Two days post-infection the selection was started with 300 μg/ml of G418 in RPMI1640. In order to confirm the successful infection we performed PCR as described before. We also extracted total RNA and did RT-PCR to assess the expression of TK gene.

Test of cell sensitivity to GCV

We planted gene-modified pancreatic cell SW1990/TK in 96-well (Nunc) plates with 5 000 cells/well. The next day, GCV was added at a concentration ranging from 0 to 500 μg/ml. five days later, cell survival rate (SR) was determined using routine MTT-method, absorbance (A) values were read on a Bio-Rad micro-plate auto-reader at 490 nm wavelength. Survival percentage was determined by ratios of absorbance values from test conditions over absorbance values from non-infected cells. SR=(A value of the test well ÷ A value of the control)×100%.

Study of bystander effect

SW1990 and SW1990/TK cells were seeded at a total density of 10 000 cells/well in 96-well plates with various proportions. GCV was added at a concentration of 50 μg/ml. Then MTT assay was performed as routine protocols, each ratio was tested at least three times.

Statistical analysis

Uni-variate two-sided analysis of matched *t*-test for dependent samples was used, the dose-response curve was obtained using Microsoft Excel.

RESULTS

Through PCR, we obtained a 404 bp long fragment, which was the expected product of HSV-TK gene (Figure 1). From SEM, we can see easily that the cell surface of transfected PA317 had a lot of progeny viruses compared with their parent cells (Figure 2). These confirmed that TK gene was successfully integrated into the genome of packaging cell PA317, which was secreting virion progeny steadily, so we named it as PA317/TK. The virus titer was 40 000 CFU/ml.

Both PCR and RT-PCR confirmed the successful infection and expression of TK gene in gene-modified SW1990 (RT-PCR product electrophoretogram see Figure 3), and we named this cell as SW1990/TK.

Our experiment demonstrated that transgenic SW1990/TK cells were sensitive to prodrug GCV compared to SW1990 (t=4.15, n=10, P<0.0025). With increase of GCV concentration, SW1990/TK presented typical morphological changes of apoptosis and cell death such as nuclear condensation and oligonucleosomal DNA fragmentation, and finally lyses (Figure 4). The survival rate decreased sharply, especially at 0.5 to 50 μg/ml GCV, while the growth of SW1990 cells was not affected. At the same time, we found that the growth of unmodified cancer cells was inhibited when the concentration was more than 100 μg/ml, as seen in Figure 5.

SW1990/TK exhibited a "bystander effect" when mixed with TK-negative cells at different ratios. From Figure 6 we can see that the cell survival rate was 40%, 20% and 0% when there was 15%, 30% and 80% of SW1990/TK cells in all, the inhibitory rate (IR=1-SR) was 60%, 80%, 100% respectively. Apparently the IR was much higher than the percentage of SW1990/TK, which reflected the bystander effect.

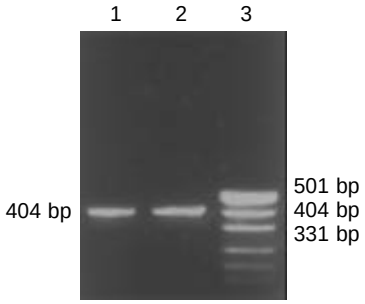

Figure 1 Electrophoretic results of PCR products. 1. GINaTK, 2. PA317/TK, 3. Marker (puc19DNA/MSP I).

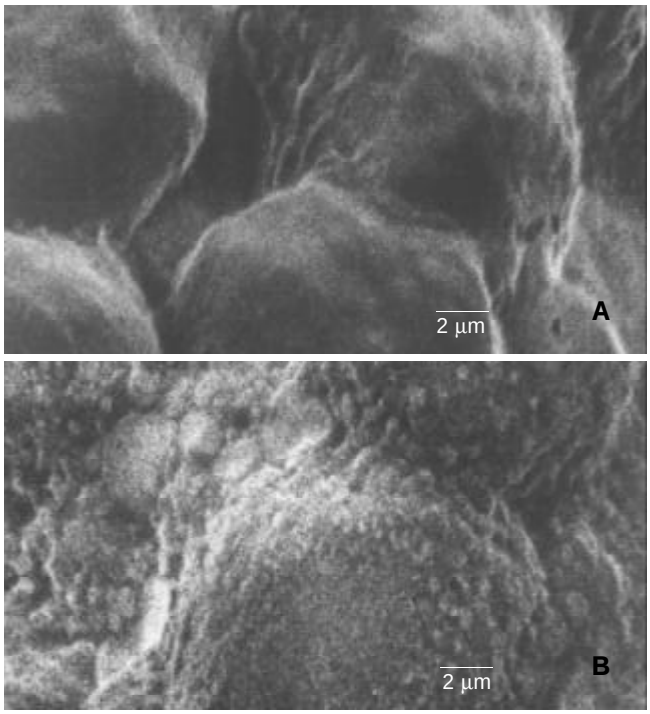

Figure 2 Results of scanning electron microscop. A: The surface of PA317 cell are very smooth, B: There are a lot of bumps on the surface of PA317/TK cells because these gene- modified cells are secreting virus particles.

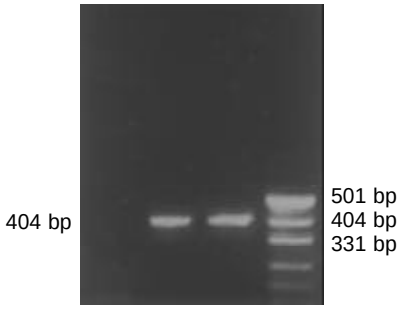

Figure 3 Results of RT-PCR. 1. SW1990, 2. SW1990/TK, 3. GINaTK, 4. Marker.

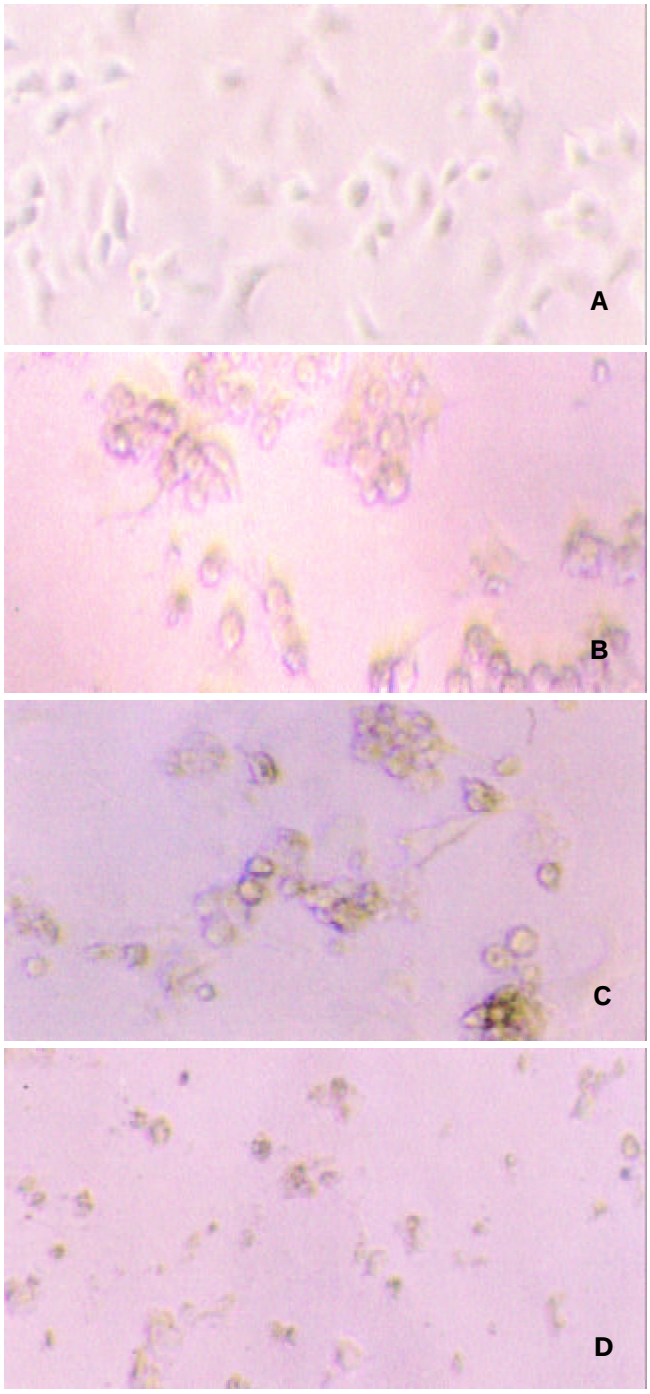

Figure 4 Typical morphological changes in SW1990/TK cells when exposed to GCV (50 μg/ml). A: SW1990/TK cells without GCV. B: Two days after adding 50 μg/ml of GCV, cells became round and smaller, losing their normal morphology. C: Four days after adding GCV, cells gathered to balls. D: Five days later, cells clasped into small fragments.

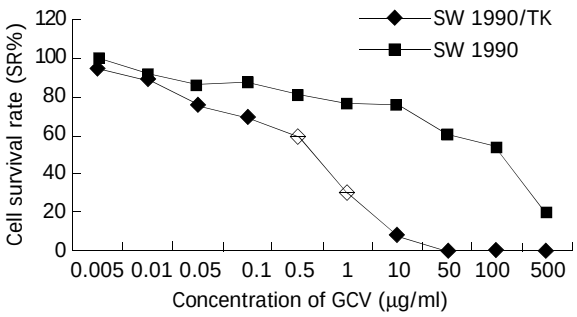

Figure 5 Sensitivity of SW1990/TK to GCV.

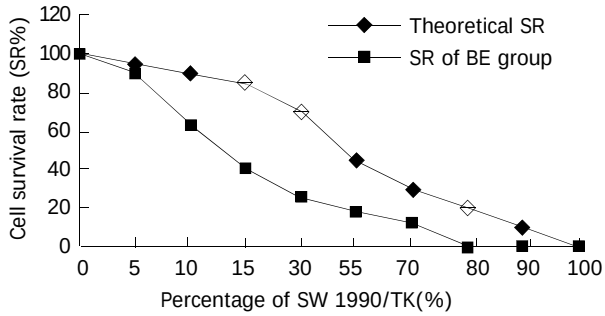

Figure 6 Bystander effects of SW1990/TK cell line.

DISCUSSION

Pancreatic cancer has an extremely poor prognosis due to lack of early diagnostic and therapeutic approaches, few are suitable for surgery and respond to chemo-radiation therapy, mainly because of its silent course and explosive fatal outcome. Most patients had locally advanced or metastatic diseases at the time of diagnosis and were therefore not amenable to resection, whilst chemotherapy and radiotherapy were by and large ineffective[11]. HSV-TK/GCV suicide gene therapy system has been widely studied these years, and is a promising approach to tumor therapy, but its use in human pancreatic cancer has been limited.

In our study, the retrovirus was used as a target gene vector. Among various vectors, retrovirus-mediated gene transfer is restricted to cells that are proliferating and synthesizing DNA at the time of infection but not nondividing cells. It is suitable to gene transfer of malignant cells with rapid proliferation and can improve the targeting of gene transfer. With 40 000 CFU/ml virus titer, it transfers target gene effectively.

Besides the advantage of retrovirus, HSV-TK/GCV suicide gene therapy system shows another superiority: bystander effect. Both the transduction efficiency and bystander effect are essential factors for the success of the anti-tumor effect of HSV-TK and prodrug GCV suicide gene therapy system. Bystander effect is described when nontransduced or genetically unmodified cells are killed as the result of enzyme-prodrug activation during the death of genetically modified tumor cells transduced with a suicide gene. The "bystander effect" greatly amplifies the efficacy of HSV-TK/GCV gene therapy for cancer in which only a fraction of the cells are targeted. The *in vitro* bystander effect in C6/C6-TK5 co-culture was highly significant, the presence of only 5% of C6-TK5 cells led to an overall 78% decrease in cell survival after 5 days of GCV treatment[12]. In our experiment, 15% of SW1990/TK cells could lead 60% cells to death. However, the mechanism of bystander effect is still controversial. Some investigators suggested that intercellular communication was essential for the bystander effect. The correlation between gap junction communication (GJIC) and the extent of bystander effect suggested a role of GJIC in mediating the bystander effect, which could provide a useful system for selective killing of gene-modified tumor cells[13]. Some provided evidences for a role of cell membrane in signal pathways leading to bystander effect.

As we all know, everything has two sides. Inevitably, this system had its own side effect. The most common side effects of GCV in immunocompromised patients were leukemia (7-42%), thrombocytopenia (8-57%), and abnormal liver-function tests (2%). However, side effects in patients with intact immune system have not been reported[14]. As we can see in Figure 5, once the dose of GCV exceeded 500 μg/ml, it did harm to unmodified cells, so control of the dosage of drug administration is important in clinical practice.

The current trend of gene therapy for tumor is to combine

two or even more approaches in order to improve its anti-tumor effect and reduce its side effects. The combination of both suicide systems of cytochrome p450 2b1 (CYP2B1)/CPA and HSV-TK/GCV *in vitro* resulted in a potentiation of the killing effect. This suggested that in order to achieve a potentiation in cell killing when two suicide systems were combined, co-expression of both genes in the same tumor cell would be necessary[15]. Gene therapy with p53 and K-ras modulated herpes viruses might become a palliative treatment option and could be used easily by regional chemotherapy techniques[1]. Pancreatic ductal adenocarcinomas (PDACs) could overexpress various cell-surface tyrosine kinase receptors, including type I high-affinity fibroblast growth factor receptor (FGFR-1). In view of the overexpression of high-affinity FGFRs in cancer cells in PDAC, Kleeff' s findings suggested that combined use of AdTK, ganciclovir, and FGF2-Fab' might ultimately be a promising therapeutic approach in a subgroup of patients with PDAC[16].

Besides suicide gene therapy system, there are many other approaches for pancreatic cancer therapy. Based on the relative uniformity in its molecular abnormalities, about 80-90% of tumors have dominant oncogene K-ras mutations and disruption of p16/RB tumor suppressor pathway, about 60-75% have p53 mutations, and more than 50% have SMAD4/DPC4 disruptions, transfer of wild-type p53 and p16 could produce significant growth suppression of pancreatic cancer *in vitro* and *in vivo* [17-19]. The results of Kawakami *et al*. showed that IL-4 receptor-targeted cytotoxin represented a potent agent that might provide an effective therapy for pancreatic cancer[20]. Yan and his group found that adenovirus-mediated E2F-1 gene transfer could sensitize melanoma cells to some chemotherapeutic agents, particularly topoisomerase II poisons *in vitro* and *in vivo*. These results suggested a new chemosensitization strategy for melanoma gene therapy[21]. With the discovery of RNA-interference, some researchers suggested it might be used in tumor therapy[22-24].

Our results indicated that gene modified tumor cells SW1990/TK were sensitive to antivirus prodrug GCV, and showed a remarkable bystander effect on killing tumor cells. In conclusion, HSV-TK/GCV suicide gene therapy system is a promising approach for the treatment of pancreatic cancer. As increasingly more researchers focus on the diagnosis and therapy of this lethal malignancy, we believe that some effective ways would be discovered for clinical treatment of pancreatic cancer.

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• CLINICAL RESEARCH •

Striking elevation in incidence and prevalence of inflammatory bowel disease in a province of western Hungary between 1977-2001

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Abstract

AIM: An investigation into inflammatory bowel disease and colorectal cancer in Veszprem Province was conducted from 1977 to 2001.

METHODS: Both hospital and outpatient records were collected and reviewed comprehensively. The majority of patients were followed up regularly.

RESULTS: The population of the province was decreased from 386 000 to 376 000 during the period. Five hundred sixty new cases of ulcerative colitis (UC), 212 of Crohn's disease (CD), and 40 of indeterminate colitis (IC) were diagnosed. The incidence rates increased from 1.66 to 11.01 cases per 100 000 persons for UC, from 0.41 to 4.68 for CD and from 0.26 to 0.74 for IC. The prevalence rate at the end of 2001 was 142.6 for UC and 52.9 cases per 100 000 persons for CD. The peak onset age in UC patients was between 30 and 40 years, in CD between 20 and 30 years. A family history of IBD was present in 3.4 % in UC and 9.9 % in CD patients. Smoking increased the risk for CD (OR=1.94) while it decreased the risk for UC (OR=0.25). Twelve colorectal carcinomas were observed in this cohort, the cumulative colorectal cancer risk after 10 years in UC was 2%, after 20 years 8.8%, after 30 years 13.3%.

CONCLUSION: The incidence and prevalence rates of IBD have increased steadily in Veszprem Province, now equivalent to that in Western European countries. Rapid increase in incidence rates supports a probable role for environmental factors. The rate of colorectal cancers in IBD is similar to that observed in Western countries.

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INTRODUCTION

The pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD) has only been partly understood. Inflammatory bowel disease (IBD) is a multifactorial polygenic disease with probable genetic heterogeneity. Based on this hypothesis, the disease may develop in a genetically predisposed host as a consequence of disregulated immune response to environmental factors, in particular enteric antigens, resulting in continuous immune-mediated inflammation[1,2]. However, the disease phenotype may be affected by various other factors.

IBD represents an important public health problem, as it tends to afflict young people and has a protracted and relapsing clinical course, affecting education, working abilities, social life and quality of life.

Several studies have been done on the epidemiology of IBD[3,4]. The geographical incidence of IBD varies considerably, the highest incidence rates were reported in Northern and Western Europe as well as North America, whereas they were lower in Africa, South America and Asia, including China[5]. It is more common in developed, more industrialized countries, urban residency seems to be a risk factor. The incidence rate of UC varies greatly between 0.5-24.5/100 000 inhabitants, that of Crohn's disease between 0.1-11/100 000 inhabitants worldwide.

Previous studies in Europe suggested that the incidence was decreased from North to South[6,7], but in the early nineties the European IBD Study Group found comparable rates in Southern and Northern Europe[8]. This tendency may be explained by the relative stable incidence in previous high incidence areas, whereas in previous low incidence areas the incidence rose continuously. A further difference is that the previously reported predominance of UC is diminishing, as CD is becoming more prevalent. The reported average incidence of UC in Europe was 10.4, that of CD was 5.6.

Few data have been available about the frequency of IBD in the Eastern European countries. In the early eighties, Vucelic *et al*.[9,10] conducted a prospective survey on the incidence of IBD in Zagreb, Croatia. They reported an incidence rate of 1.5/100 000 inhabitants in UC and 0.7 in CD. Similarly low incidence of UC (1.7) and CD (1.4) was reported in another prospective study in Estonia[11]. Nagy *et al*.[12] reported epidemiological data in IBD from the early 60's to the late 80's among Hungarians. The reported incidence rate was 3.6 in UC and 1.0 in CD. These data were based mainly on hospital reports. No population based survey has been reported.

The elevated colorectal cancer (CRC) risk in patients with long standing IBD has been widely accepted since Crohn's original report. Recent observations suggested that the prevalence of CRC was also higher in CD[13]. The magnitude of the risk complicating IBD varied greatly in different geographic areas and in different studies. From Eastern Europe we found only one publication about CRC in IBD[14].

Recently we reported on the prevalence of extraintestinal manifestations in a large IBD cohort in a long-term follow-up study[15]. The aim of the present study was to determine the incidence and prevalence of IBD and the main epidemiological features of the disease in a province of Hungary in a population based survey.

MATERIALS AND METHODS

Demographical data

Veszprem Province is located in the Western part of Hungary. The province consists of both industrial and agricultural regions.

The number of permanent population was relatively stable, with a slight decrease from 386 462 to 376 211 from 1980 to 1998 (Table 1). The rate of Gypsies is below the Hungarian average (2.5%), few Jewish people live in the province. The ratio of urban/rural residence was also relative stable (the data in 1991 were used for comparison, Table 2).

There are 7 general hospitals in the province, each is staffed by at least one gastroenterologist or internist with special interest in gastroenterology. The majority of the patients (74% of UC patients and 94% of CD patients) were followed-up in the Csolnoky F. Province Hospital in Veszprem. The main data sources were the hospital records, outpatient clinical reports, endoscopical, radiological and pathological reports collected from the Internal Medicine Department, Surgery Department, Outpatient Units and family doctors. Both inpatients and outpatients permanently residing in the investigated area were included in the study. Most of the patients were followed up regularly. Diagnoses (based on hospitalization records, outpatient visits, endoscopic, radiological and histological evidence) generated in each hospital and outpatient unit were reviewed thoroughly, using the Lennard-Jones criteria[16].

Cases having another readily identifiable cause of colitis, such as infectious colitis (including pseudomembranous) were excluded. Anticoncipient or NSAID associated colitis and ischemic colitis cases were also excluded. UC cases with involvement outside the colon, with the exception of "backwash ileitis", were excluded. In some cases the final diagnosis was made years after the beginning of symptoms.

The term indeterminate colitis (IC) was first described by Price in 1978[17] for cases operated on because of non-differentiable fulminating colitis. Today it has been used for IBD colitis cases, when the data were insufficient to differentiate between UC and CD[8].

Before the early eighties the diagnosis was based mainly on rectoscopy, histology, barium enema and upper GI series. Later colonoscopy, double-contrast barium enema and selective enterography were the basic diagnostic methods, CT and in some cases leukocyte scintigraphy were also more frequently performed to help make a more accurate diagnosis (*e.g.* location, activity and complications).

The location in UC was determined by colonoscopy and/or double-contrast colonography based on the macroscopic picture. Patients were classified according to the greatest known extent. In mild to moderately severe active UC cases the location was determined during the active phase, in more severe disease shortly after the active period.

The study was retrospective. Data of IBD patients were summarized yearly, in some cases the diagnosis was changed after re-evaluation. IBD patient data were collected every year from the 7 general hospitals and gastroenterology outpatient units. The provincial IBD register data were centralized in Veszprem, which is the secondary referral center for IBD patients in the province. Patients diagnosed in the same calendar year were included in the incidence calculations. All the residents and IBD patients permanently residing in the province on the 31st of December 1991 and 2001 were included in the prevalence calculations (including patients who had moved to Veszprem Province after diagnosis).

Regular colonoscopic dysplasia-cancer surveillance program with multiple biopsies was carried out in patients with extensive colitis after 8 years, with left-sided colitis after 12 years.

The source of demographic data was the Hungarian Central Statistical Office (KSH).

Table 1 Population of Veszprem Province between 1980-1998

Years	Total	Women	Men
1980	386.462	194.855	191.607
1990	379.246	192.867	186.462
1998	376.211	192.296	183.915

Male / female ratio in 1990: 0.966.

Table 2 Proportion of patients according to places of residence (in 1991) (OR: Odds ratio)

	Urban (*n*)	Rural (*n*)	Total (*n*)
Veszprem Province	208.284	173.089	381.373
Ulcerative colitis	339	221	560
	OR: 1.27		
	(95% CI: 1.07-1.52)		
Crohn's disease	122	90	212
	OR: 1.13		
	(95% CI: 0.85-1.49)		

Statistical methods

For statistical comparison of the data, Statistica 6.0 (Statsoft Inc., USA) was used. Odds ratio (OR) was calculated for potential factors that influenced the prevalence of IBD, including residence (urban versus rural) and smoking habits. For comparisons within group ANOVA analysis with Scheffe post hoc test was used. Yates-corrected Chi-square analysis was performed to compare differences in incidence according to the location of UC or CD during the observed period. Results were expressed as mean±SD if otherwise not stated (NS=not statistically significant).

RESULTS

Incidence

During the observation period 560 new patients with UC (M/F: 288/272, ratio: 1.058) and 212 new patients with CD (M/F: 108/104, ratio: 1.038) were diagnosed in Veszprem Province.

The mean incidence rate for UC was 5.89 (95%CI: 2.15-9.63) cases per 100 000 persons per year. The sex-standardized incidence appeared slightly higher in men (6.19, 95%CI: 2.30-10.08) than in women (5.64, 95%CI: 2.39-8.89), however, the difference was not statistically significant. For the calculation of the sex standardized incidence rates, gender specific population data of Veszprem Province were used (Hungarian Central Statistical Office, KSH). In CD the average 25-year incidence was 2.23 (95%CI: 0.5-3.96), in men: 2.31 (95%CI: 0.64-3.98 and in women: 2.17 (95%CI: 0.34-4). Indeterminate colitis was diagnosed in forty cases (M/F: 22/18), the mean incidence rate was 0.42 cases per 100 000 persons per year.

The incidence of IBD in 5-year intervals is shown in Figure 1. A sharp increase in incidence of UC was observed from 1.66 during 1977-1981 to 11.01 during 1997-2001 (in men from 1.77 to 11.96 and in women from 1.54 to 10.09). For CD, a similar tendency was observed. The incidence rose from 0.41 to 4.68, in men from 0.21 to 5.76, in women from 0.41 to 3.64. An almost continuous rise in incidence was observed for both UC and CD. The incidence of IC also rose during the observed period (from 0 to 0.74).

In contrast to UC, for which the highest incidence rate recorded was in 2001 in both sexes (in men 16.85, in women 11.96), for CD the peak incidence recorded in men was 9.24 in 1998, whereas in women it was 5.72 in 2001.

The ratio of UC/CD incidence rates decreased from 4.05 to 2. 35 during the observed periods.

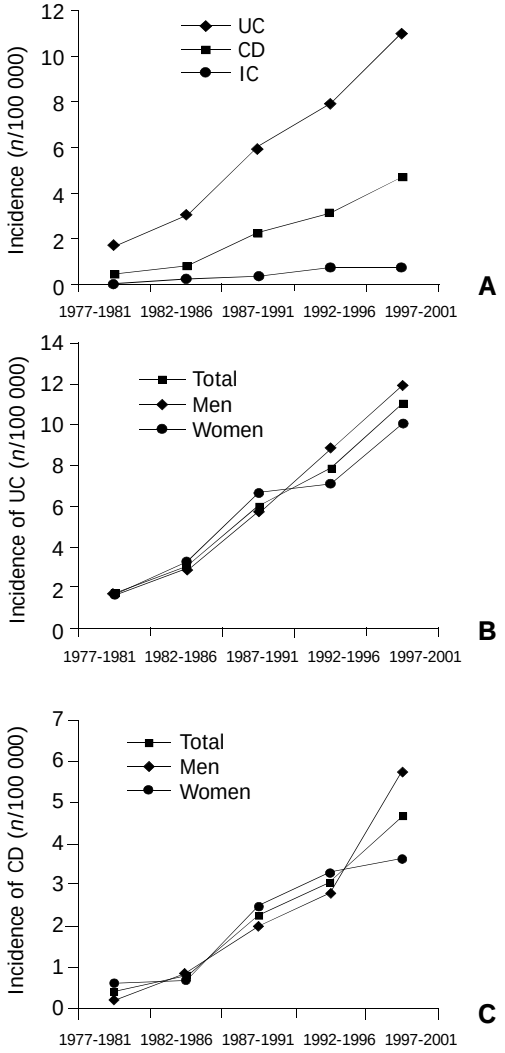

Figure 1 Average and sex-specific incidence of UC, CD and IC in Veszprem Province between 1977-2001.

Age standardized incidence rates

The mean age at diagnosis was 31.7±12.8 years for CD, 38.9±15.5 years for UC and 36.8±13.9 years for IC. For the calculation of age standardized incidence rates, age specific population data of Veszprem Province were used (Hungarian Central Statistical Office, KSH). We observed only one peak incidence. For UC the highest incidence rate was in 31-40-year olds, but the 21-30-year olds incidence rate was in the same range (10.96 *vs* 9.26). For CD the peak incidence was in the 21-30-year olds (Figures 2A and B). The youngest UC patient was diagnosed at the age of 9 years, while the oldest patient just passed 80 years at the time of diagnosis. The youngest CD-diagnosed-patient was 12, the oldest 80 years old.

Prevalences

All the patients permanently living in Veszprem Province were included in the prevalence calculation (even patients diagnosed before 1977, 33 UC cases and 3 CD cases were not included as incidence cases).

On the 31st of December 1991, the prevalence of UC was 59.2 cases per 100 000 persons, that of CD was 17.1 cases per 100 000 persons. On this day the prevalence of IC was 2.9/ 100 000. The prevalences were significantly increased by the 31st of December 2001. For UC the prevalence was 142.6, while for CD 52.9 cases per 100 000 persons.

The urban/rural ratio was 1.205 in general population in 1991, which was relatively stable during the observed period. Urban residency seemed to increase the risk of UC (OR: 1.27), but it did not affect significantly the risk for CD (Table 2).

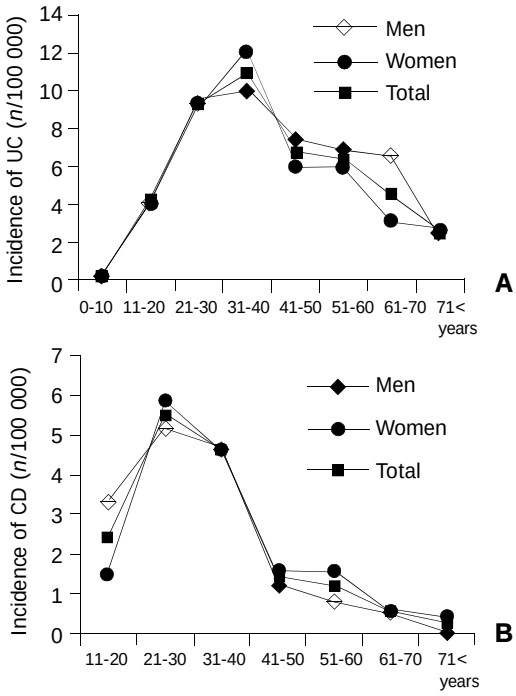

Figure 2 Age and sex-specific incidence of UC and CD in Veszprem Province between 1977-2001.

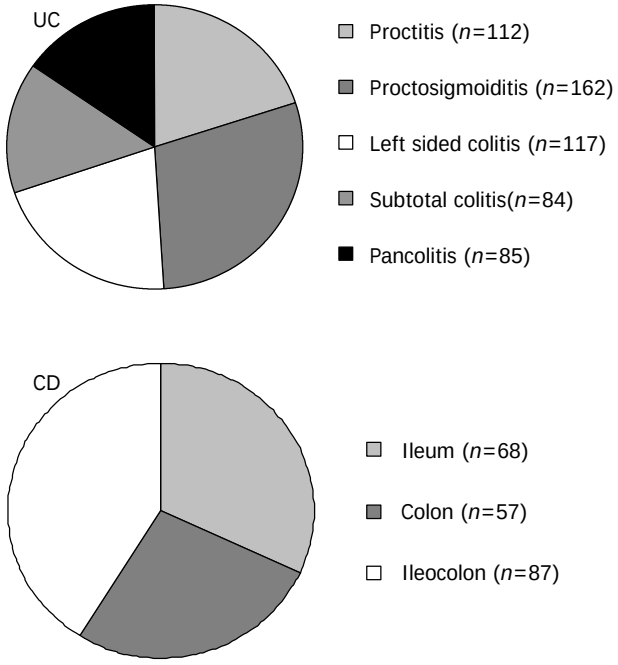

Figure 3 Location of UC or CD (*n*=number of patients).

Location and disease behavior

According to our information 112 of the UC patients had proctitis, 162 proctosigmoiditis, 117 left sided colitis, 84 subtotal and 85 pancolitis (Figure 3A). The age at diagnosis did not affect the location of the disease.

Location of UC did change in the past 25 years, proctitis became more prevalent in the last decade compared to the first 10 years (8.6% *vs* 24.0%, *P*=0.002), while pancolitis cases

were relatively less frequent (39.8% *vs* 27.8%, *P*=0.034). The percentage of left sided colitis did not change (51.6% *vs* 48.2%).

The Vienna classification[18] defines CD patients according to the age at onset, location and disease behavior. In CD ileal disease (L1) was found in 32.1%, colonic (L2) in 26.9% and ileocolonic (L3) in 41.0% of the patients (Figure 3B). Location of CD did also change in the observed period. There was a tendency of colonic location becoming more frequent in the last decade compared to the first one, while the percentage of ileocolonic cases was decreased (L1: 19.2% *vs* 24.1%, *P*=NS, L2: 19.2% *vs* 36.5%, *P*=NS, L3: 61.5% *vs* 39.4%, *P*=0.05). The most commonly associated location was perianal (48 cases). All the patients with upper gastrointestinal location (L4) had lower gastrointestinal involvement, and were classified according to this location (L1-3). In 5 cases the jejunum, in 2 the duodenum, in 5 the stomach and in 2 the esophagus were also affected. Only histologically proven cases were included in the calculation of associated locations. There was no correlation between the location and age at diagnosis.

According to the disease behavior 63 of our CD patients were defined as non-stricturing, non-penetrating (B1), 54 as stricturing (B2) and 95 as penetrating (B3). Fifty-three patients of the 95 penetrating cases had parallel strictures.

In UC group 6.4% of the patients had at least one bowel operation, a much higher frequency was observed in CD (60%, Table 5). There was an association between disease location and the frequency of operations (L1=0.98, L2=0.21, L3=1.93 operations on average, *P*<0.01 among all the groups, ANOVA-Scheffe post hoc test).

Familial incidence

Of the UC patients 19 had one or more close relatives with confirmed UC or CD (Table 3). In CD patients IBD was apparent in 9.9% of the families. The occurrence of IBD was more pronounced in families of CD patients with stricturing disease than in those of CD cases with non-stricturing, non-penetrating disease (B1: 6.6% (4/60), B2: 24.5% (13/53) and B3: 11.7% (11/94), *P*=0.04).

Table 3 Familial occurrence of IBD

Relationship	UC (*n*=560)	CD (*n*=212)
First-degree relative with IBD	17	15
Second-degree relative with IBD	2	6
Familial IBD	3.4 %	9.9%

Smoking

Sixty-seven UC patients (14.3%) were smokers at the time of diagnosis, while the rate of ex-smokers and nonsmokers was 18.4% and 67.3%, respectively (Table 4). In CD patients 102 were smokers at the time of diagnosis (50.5%, which was higher than the Hungarian average of 40.5%, 1991 data, KSH). Among the patients with CD, 13 ex-smokers and 87 nonsmokers were identified. Smoking decreased the risk for UC by 75%, while it almost doubled the risk for CD (OR: 1.94, 95% CI: 1.46-2.59).

Colorectal carcinoma

Colon tumor was observed in 10 patients with UC (Table 6). The average duration of IBD at the diagnosis of colorectal cancer was 18.4 years. The average age at the diagnosis of CRC was 53.4 (27-65) years, 15 years younger than the age of patients with non-IBD CRC at diagnosis (68.2 years, 2000 data, KSH). The male/female ratio was one (5/5). Seven out of the ten patients had pancolitis. Two colorectal cancers occurred in CD patients with a short follow-up (3 and 6 years) period. The location of CD was colonic without ileal involvement. No family history of IBD was apparent in IBD cases with CRC. Only one out of the twelve patients with IBD and CRC had a family history of CRC.

The cumulative risk for colorectal cancer in patients with UC for 10 years was 2%, for 20 years 8.8%, and for 30 years 13.3%, respectively.

Table 4 Smoking habits in IBD patients at presentation

	UC *n* (%)	CD *n* (%)
Non-smoker	315 (67.3%)	87 (43.1%)
Ex-smoker	86 (18.4%)	13 (6.4%)
Smoker	67 (14.3%)	102 (50.5%)
No data	92	10
Odds ratio for smoking	0.25 (95% CI: 0.19-0.32)	1.94 (95% CI: 1.46-2.59)

(Prevalence of smoking in Hungary in 1991: 40.5%).

Table 5 Surgery in IBD

Surgery in UC	Surgery in CD
• Resection: 8	Not operated: 86 (40.6 %)
• Proctocolectomy: 28	Operated: 126 (59.4 %)
- J Pouch: 25	• 1 operation: 63 (29.7 %)
	• 2-3 operations: 50 (23.6 %)
	• 4-8 operations: 13 (6.1 %)

Table 6 Colorectal carcinoma in IBD

Carcinomas	Age at diagnosis	Duration of IBD	Location of IBD	Location of tumor
UC (*n*=10)	35 yrs (20-56 yrs)	18.4 yrs (9-33 yrs)	Pancolitis: 7 Left sided colitis: 3	Ascending: 2 Transverse: 4 Descending: 2 Rectum: 2
CD (*n*=2)	24 and 45 yrs	3 and 6 yrs	Right colon: 2	Transverse: 2

DISCUSSION

The incidence of IBD varied greatly worldwide. Genetic and environmental factors were assumed to play a significant role in the etiology of the disease[1,2]. The role of genetic factors was supported by ethnical and familial differences and also by twin studies[19-22], while the differences in incidence rates among various geographical areas suggest a role for certain environmental factors. There has been an important change in incidence of IBD in the last few decades. In high incidence countries in Western Europe the incidence rate remained relatively stable, while in previous low incidence areas[9,12] as supported by our data as well, the disease has become more common.

The continuous increase in incidence rates of both UC and CD observed in this study raises further questions. What could be the cause of this change? In the first years the lower incidence rates could be explained partly by the use of less up-to-date diagnostic procedures (*e.g.* the relative low availability of selective enterography or colonoscopy). It is possible that better awareness, either by physicians or by patients, may result in the diagnosis of mild cases that previously might have gone unnoticed. There has also been an important change in patients' behavior in Hungary, as patients tend to seek medical advice more often and with milder symptoms than they did two decades ago. We believe that the increase in the incidence of IBD in the second part of the observed period is real and not

solely due to an improved diagnosis or more extensive search. This idea is also supported by the increase in severe cases that could only be interpreted as real. Our incidence rates in UC (11.01) and CD (4.68) in the last five-year period (1997-2001) were in the range as previously observed in high incidence Nordic countries[7,8,23,24] and they were much higher than the rates reported in Hungary two decades ago[12].

Most studies reported the peak onset of both UC and CD at late puberty - early adulthood period. In some studies a second peak of onset was observed in 50-70 year-olds[25]. The EC-IBD study[8] reported one peak onset in CD in both sexes around the age of 20 (incidence rate in men was around 6.0 and in women 7.7) followed by a continuous decrease. In UC the peak incidence was observed in both sexes in 25-30-year olds (incidence rate was 11.2 in men and 10.7 in women), followed by a continuous decrease in women, while the incidence remained relatively stable in men. In the present study we observed one peak incidence in UC in 30-40 year olds (in men 10.0 and in women 12.1) and also in CD in 20-30 year olds (in men 5.2 and in women 5.9).

IBD could affect both sexes almost equally, with a slight predominance of women in CD, and a male/female ratio ranging from 1.1 to 2.0 in UC[5,8,23]. In our present study the male to female ratio was almost similar with a slight male predominance.

In UC the proportion of extensive colitis was higher, while the percentage of proctitis cases was lower than those in the recent EC-IBD study[8]. Location of UC cases only mildly changed in the observed period. The percentage of pancolitis cases was decreased, in contrast, procitis became more prevalent. However it was still less frequent than observed in the Western-European epidemiological studies[8].

In CD ileal disease was found in one third of the patients, and ileocolonic in 41.0% of the patients. A recent study reported a higher frequency of ileocolonic disease while the percentage of ileal disease was lower[8]. In the current study colonic involvement was observed in almost two-thirds of the patients, and there seemed to be a shift from ileal to colonic location during the observed period.

The rapid increase in incidence rates might support a role for possible environmental factors[26]. Diet, as a luminar antigen was thought to be an important factor in the pathogenesis of IBD[1,27]. In the last two decades there has been a change in the lifestyle in Hungary, as the standard way of living including the diet became more "Western". Other possible environmental factors, such as perinatal events, infections in childhood or measles have not been investigated in this study[26,28,29]. Measles vaccination is universal in Hungary, and the disease is very rare. The birth rate is one of the lowest ones in Europe. Early childhood hygiene is developed, supporting the "oversheltered child" theory[30].

One of the most important environmental factors considered in the etiology of IBD was smoking[1,31]. In concordance with previous data we could identify smoking as a protective factor in patients with UC (OR: 0.25). In contrast, smoking increased the risk for CD almost 2-fold.

The percentage of operations in UC in our study was below the 10-20% rate reported in most series. The reason for this could be the more conservative practice in Hungary and also the greater resistance of patients to surgery. The majority of the operations were proctocolectomies with ileal pouch anal anastomosis (IPAA). The proportion of surgically treated patients in CD was similar to that previously published (60-80%). Almost one third of the CD patients had multiple operations.

There is an increased risk of CRC in IBD, but the reported incidence rates varied greatly. Earlier studies from tertiary referral centers reported rather high cumulative incidence rates of CRC (>10% at 20 years, >30% at 30 years). However, it might reflect referral biases, because of overrepresentation of more serious diseases in these centers that did not represent the usual spectrum of IBD[32]. In population-based studies, that followed a larger number of patients for a long time, in a certain area, lower CRC rates were reported (5.5% at 20 years, 13.5% at 30 years). The rate of colorectal cancers in our study was similar to that reported in previous population-based studies.

The two most important risk factors were the disease duration and extent (in CD the colonic involvement)[32,33]. Other factors involved are early age at onset, pharmacotherapy, disease activity (relapsing-chronically active, number and severity of relapses), smoking habits, family history of CRC, the presence of primary sclerosing cholangitis, backwash ileitis. In our study the UC patients with CRC were non-smokers while the two CD patients were smokers during the follow-up, however our numbers were relatively small to make a reasonable conclusion.

Prophylactic colectomy, regular mesalamine and folic acid treatment, and colonoscopic surveillance might reduce the elevated CRC risk[34,35]. In our study all the patients with IBD and CRC received 5-ASA. In concordance with previous results CRC was diagnosed in only one out of 32 patients with UC and in none of 66 patients with CD taking azathioprine, such azathioprine did not seem to increase cancer risk. Colonoscopic surveillance was the most widely used method to reduce CRC risk, but evidences of its benefits were controversial[36,37]. We also performed colonoscopic surveillance, which might have reduced cancer risk.

There is an additional increased risk of both colorectal and hepatobiliary malignancies in the subset of patients with primary sclerosing cholangitis (PSC) and IBD[38]. In our cohort three colorectal carcinomas developed in the 13 PSC patients, showing an increased risk in this subgroup. The reason for the short interval (3 and 6 years) between the diagnosis of CD and colorectal cancer is not clear. One may hypothesize that the asymptomatic course of IBD may be responsible for the early occurrence of colorectal cancers.

In conclusion, the incidence and prevalence have been steadily rising in the last 25 years and Now it reaches the levels reported in Western European countries. We observed no gender differences. There was a relative high incidence of colorectal cancers in IBD, similar to Western countries. The cause of the continuous rapid increase in the incidence of IBD is unknown, but the incidence supports a possible role for environmental (*e.g.* diet, lifestyle) factors.

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• CLINICAL RESEARCH •

Comparative observation on different intervention procedures in benign stricture of gastrointestinal tract

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Abstract

AIM: To determine the most effective intervention procedure by evaluation of mid and long-term therapeutic efficacy in patients of stricture of the gastrointestinal tract (GIT).

METHODS: Different intervention procedures were used to treat benign stricture of GIT in 180 patients including pneumatic dilation (group A, *n*=80), permanent (group B, *n*=25) and temporary (group C, *n*=75) placement of expandable metallic stents.

RESULTS: The diameters of the strictured GIT were significantly greater after the treatment of all procedures employed ($P<0.01$). For the 80 patients in group A, 160 dilations were performed (mean, 2.0 times per patient). Complications in group A included chest pain (*n*=20), reflux (*n*=16), and bleeding (*n*=6). Dysphagia relapse occurred in 24 (30%) and 48 (60%) patients respectively during 6-and-12 momth follow-up periods in group A. In group B, 25 uncovered or partially covered or antireflux covered expandable metallic stents were placed permantly, complications included chest pain (*n*=10), reflux (*n*=15), bleeding (*n*=3), and stent migration (*n*=4), and dysphagia relapse occurred in 5 (20%) and 3 patients (25%) during the 6-and-12 month follow-up periods, respectively. In group C, the partially covered expandable metallic stents were temporarily placed in 75 patients and removed after 3 to 7 days via gastroscope, complications including chest pain (*n*=30), reflux (*n*=9), and bleeding (*n*=12), and dysphagia relapse occurred in 9 (12%) and 8 patients (16%) during the 6-and-12 month follow-up periods, respectively. The placement and withdrawal of stents were all successfully performed. The follow-up of all patients lasted for 6 to 96 months (mean 45.3±18.6 months).

CONCLUSION: The effective procedures for benign GIT stricture are pneumatic dilation and temporary placement of partially-covered expandable metallic stents. Temporary placement of partially-covered expandable metallic stents is one of the best methods for benign GIT strictures in mid and long-term therapeutic efficacy.

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INTRODUCTION

Benign stricture of gastrointestinal tract (GIT) is caused by postsurgical anastomoses, ingestion of corrosive agents, simple sclerosis after radiation therapy for tumors, digestive ulcer and functional disturbances, which involve different sites including esophagus, stomach, duodenum, colon and rectum. From July 1994, 180 patients with benign GIT stricture were treated with intervention procedures. Our experiences and follow-up data are reported herein.

MATERIALS AND METHODS

Materials

Our cohort comprised 180 patients with benign GIT stricture (101 males, 79 females; age, 12 to 78 years, mean 48.7 years). The subjects were divided into three groups according to the intervention procedures used: 80 patients with pneumatic dilation (group A), 25 with permanent uncovered or partially covered or antireflux covered metallic stent dilation (group B), and 75 with temporary partially covered metallic stent dilation (group C). Among the 180 patients, 8 had simple sclerosis stricture after radiation therapy for esophageal carcinoma, 132 had achalasia, 32 had esophageal and esophagogastric anastomosis stricture (complicated with anastomosis fistula in two patients), 4 had gastroduodenal anastomosis stricture, and 4 had esophageal chemical corrosive stricture. All patients were examined by barium radiography of GIT and gastroscopy before the intervention procedures.

Methods

The GIT was emptied for at least 4 h before intervention procedures. Bleeding and clotting times were examined. The devices used were as follows. The catheter was an SY dumbbell-like catheter (Sanyuan Medical Instrument Research Institute, Jinan, Shandong, China) with a length of 75 cm. The diameters upon saccule dilation were 28 mm, 30 mm, and 32 mm, and length of the saccule was 8 cm. There were two types of metallic stents, one was an imported covered Z-stent made from stainless steel wire (Wilson-Cook Medical Inc, NC, USA), the other made domestically from nitinol and uncovered or partially covered or antireflux covered (Zhiye Medical Instrument Research Institute, Changzhou, Jiangsu, China; Youyi Yijin Advanced Materials Co. Ltd, Beijin, China). The body of the partially covered metallic stents was coated with intracavity silica gel. The areas within 2 cm of both ends of the stent were not membrane covered. The stents were 4 to 14 cm in length and 16 to 30 mm in diameter, with one or two horns (diameter, 20 to 35 mm).

Patients for pneumatic dilation were placed in a supine or

Table 1 Incidence of complications following treatment with different intervention procedures (%)

Groups	Patient numbers (*n*)	Number (%) with pain (*n*)	Number (%) with reflux (*n*)	Number (%) with bleeding (*n*)	Number (%) with stent migration (*n*)
A	80	20 (25.0%)	16 (20.0%)	6 (7.5%)	-
B	25	10 (40.0%)	15 (60.0%)	3 (12.0%)	4 (16.0%)
C	75	30 (40.0%)	9 (12.0%)	12 (16.0%)	-

Table 2 Dysphagia relapse rate during follow-up

Group	6 months follow-up		12 months follow-up	
	Number tested (*n*)	Number (%) with DR (*n*)	Number tested (*n*)	Number (%) with DR(*n*)
A	80	24(30%)	80	48(60%)
B	25	5(20%)	12	3(25%)
C	75	9(12%)	50	8(16%)

DR: dysphagia relapse.

sitting position. Surface anesthesia was first applied to the pharynx. The guidewire was inserted through the mouth and passed through the stricture section as demonstrated by X-ray examination. The catheter with a diameter of 28 mm was introduced through the region of benign esophageal stricture via the guidewire, with the center of saccule at the most-strictured section. The saccule was injected using an injector with the dilated contrast medium or gas. Under fluoroscopy and according to the pain reaction of the patient, pressurization was applied to gradually dilate the saccule. The central portion of the saccule was dumbbell-shaped. When further pressurization flattened the surface of the saccule or when the pressure did not further change, the piston was turned off. The pressure of the saccule was maintained for 5 to 30 min. After the saccule pressure had reduced for 5 min, pressurization was again applied. Typically each treatment involved 3 to 5 dilations, and then the catheter was withdrawn. The second and third treatments with graded pneumatic dilation were carried out using dilators with diameters of 30 mm and 32 mm, respectively. In some patients, the treatment was conducted every 2 weeks until clinical symptoms disappeared.

The placement of metallic stents was performed as follows. In upper GIT, lidocaine (1%) was first sprayed (as a mist) for anaesthesia on the pharynx. Patients were placed in a sitting position or lying on the side. Applicable false tooth were removed and a tooth bracket was mounted. A 260 cm long exchange guidewire was inserted into the stomach. The stent was mounted on the propeller whose front end was coated with sterilized liquid paraffin. Guided by the wire, the propeller on which the stent was mounted was moved through the section of pathological change. Under fluoroscopic control, the outer sheath was slowly withdrawn and the stent was expanded under its own tension. After placing a stent, GIT radiography was performed to observe the patency of the GIT. In group C, 500 to 1 000 ml ice-cold water was injected via a bioptic hole under gastroscope for 3 to 7 days after stent placement, which resulted in retraction of the stent and reduced its diameter. Bioptic pliers were then used to withdraw the stent using a gastroscope. Gastroscopy was performed again to detect complications, such as bleeding, mucosa tearing, or perforation. Patients returned to the ward and consumed cold drinks and liquid food for 2 days before resuming a normal diet. It was preferable for patients to eat solid food since the natural expansion of the food reduced the retraction of the GIT. The criterion for therapeutic efficacy was the diameter of the most-strictured gastrointestinal segment before and after dilation.

For postoperative treatment of pneumatic dilation, barium radiography of the GIT was performed immediately after intervention procedure to observe the patency of the GIT and check the presence of perforations and submucous hematoma. Patients drank fluids 2 h after intervention procedure and were treated with antibiotics, antacids, antireflux drugs, and analgesics. For postoperative treatment of stent placement, barium radiography was used to observe the patency of the GIT. Patients ate semisolid food on the day following intervention procedure. Within one week after stent removal, barium radiography of the GIT was again used to observe the patency of the GIT. Patients were followed-up by telephone and out-patients after 1 month, 6 months, 1 year.

RESULTS

The diameters of the strictured GIT were significantly greater after the treatment of all procedures employed ($P<0.01$). The 80 patients in group A involved 160 dilations (mean 2.0 times per patient). Among them, five graded dilations of increasing diameters were performed in 1 patient, three in 29 patients, two in 18 patients and a single dilation in 32 patients. In the 25 patients of group B, uncovered or partially covered or antireflux covered stents were placed. Stent placement was successful in 100% of the patients. In the 75 patients of group C, 75 partially covered stents were placed and removed under gastroscope guidance 3 to 7 days after intervention procedure. The success rate of stent placement and extraction was 100%. The complications of the treatment are listed in Table 1, and the relapse rates of dysphagia are listed in Table 2.

DISCUSSION

Benign stricture of the GIT is a common complication of gastrointestinal diseases. Its causes are diverse, its treatment is usually difficult. The procedures used included surgery, bougienage, pneumatic dilation, permanent metallic and temporary metallic stent dilation, each having their own advantages and drawbacks[1–7]. Bougienage is now uncommon since it has a poor therapeutic efficacy and many complications. The use of surgery is declining due to the associated large lesion, high risk, and high relapse rate, but it is still one of the most common method of treatment. Pneumatic dilation was primarily used in the plasty of angiostenosis, and then applied gradually to other organs for its reliable therapeutic efficacy. It exhibits a remarkable therapeutic efficacy when used in benign esophageal stricture. Currently, it has been widely used in the nonsurgical treatment of benign GIT stricture. According to most authors[8-31] , the graded dilation is more effective than single dilation.

Permanent metallic stent dilation was primarily used in the treatment of malignant obstruction of the GIT, and exhibited a remarkable palliative therapeutic efficacy[32-40]. Cwikiel *et al*[2] reported an experimental and clinical study of the treatment of benign esophageal stricture with expandable metallic stents. We used uncovered or partially covered or antireflux covered stents in 25 patients of benign GIT stricture in order to reduce the possibility of stent migration. After placement of the uncovered stent, dilation of the stricture was excellent and dysphagia disappeared. Thus we achieved the treatment goal. However, the patients were accompanied by new problems including gastroesophageal reflux or biliary regurgitation, followed by occurrence of restenosis (hyperplasia of granulation tissue). Reflux could be treated with drugs, but this took a long time. Restenosis was reduced after cauterization using hot-point therapy under gastroscope guidance, but it was easy to relapse. Even though an antireflux stent was used, many unexpected results appeared. These difficulties led to dilation using temporary partially covered metallic stents. After their clinical trials, they not only produced fewer complications, but also exhibited excellent therapeutic efficacy. Now their use has been gradually accepted by clinicians.

For the temporary metallic stents, optimal placement time remains to be determined. If the therapeutic efficacy is poor, stents cannot be easily removed after a long-time placement. Usually, the stents are placed within 1 week. Cwikiel *et al*[2] placed a covered metallic stent in the esophagus of the pigs in an experimental study. One week later, granulation tissue grew and merged with the noncovered area of the stent, resulting in difficulties for removing the stent. The stent could not be removed following the placement for 10 to 14 days or longer. By our experience, stent migration occurred mostly within 1 week. Therefore, after the placement of a partially covered metallic stent, it should be extracted within 1 week. In our series, the stent was easily removed on the third to forth day, but this became quite difficult on the fifth day, and extremely difficult after 6 to 7 days. Song *et al*[3] reported the removal of a stent 2 months after its placement. In such patients the stent should be completely coated (including its outer layer) so that granulation tissue cannot grow into the lumen. However, the use of this type of stent should be limited to patients with tumor, since in patients of benign GIT stricture, it migrates easily. In terms of the degree of acceptance of patients, therapeutic efficacy, extent of tissue lesion, and incidence of complications, the best method for malignant stricture or obstruction of the GIT is the partially covered metallic stent, and for benign stricture of the GIT, graded pneumatic dilation or temporary partially covered metallic stent dilation should be recommeded[41-45].

Sixty percent of patients with the follow-up of 1 year or longer had dysphagia relapse, demonstrating that pneumatic dilation of benign stricture of the GIT had an excellently immediate therapeutic efficacy but a poor mid and long-term therapeutic efficacy. First, this was associated with the diameter of saccule. Kadakia *et al*[4] suggested that the diameter of the saccule in pneumatic dilation should be 35 to 45 mm, but the incidence of complications was very high (*e.g.*, 15% presented esophageal perforation). We used saccules with a diameter of 28 to 32 mm in order to reduce the incidence of serious complications, but the mid and long-term therapeutic efficacy was not satisfactory. Second, the therapeutic efficacy was associated with the frequency of dilation. One dilation did not produce excellent therapeutic efficacy, since it was affected by various factors such as the correct location of the saccule pressure applied to the saccule, and variations in the anatomy of GIT. The graded dilation was suggested by most authors. Third, the therapeutic efficacy was associated to the course of the disease. When the course was long, the GIT muscularis would become fleshy and lose elasticity.

Permanent uncovered or partially covered metallic stents were used in the treatment of malignant stricture or obstruction of the GIT with excellently immediate therapeutic efficacy and poor mid and long-term therapeutic efficacy. This was mainly due to tumor growth. Since uncovered or partially covered metallic stents could only provide palliative treatment for the obstruction, only by adopting a combined therapy for the tumor, can mid and long-term therapeutic efficacy be achieved. In our series, permanent uncovered or partially covered or antireflux covered metallic stent dilations were used in 25 patients of benign GIT stricture, their immediate therapeutic efficacy was excellent and the mid and long-term efficacies were unsatisfactory. The poor mid and long-term outcome for permanent uncovered metallic stent dilation was mainly due to frequent gastroesophageal reflux or biliary regurgitation and restenosis. Three uncovered stents could not be extracted after a 12-month follow-up period, and hence the cardia had to be excised with the stent and surgically reconstructed. Therefore, permanent uncovered metallic stent dilation was not suitable for patients with functional GIT stricture[46-49]. Permanent partially covered metallic stent dilation had poor mid and long-term therapeutic effects. This was mainly due to reflux and stent migration. Temporary partially covered metallic stent dilation used for benign GIT stricture resulted in excellent immediate effect, thus becoming the best method for mid and long-term therapeutic efficiencies. First, design of the stent coincided with the physiological structure of the gastrointestinal tract and the specific pathological manifestations of the benign stricture. The upper outlet of the stent was a large horn without cover, increasing stability of the stent. However, this made removal of the stent more difficult. Second, the diameter of the stents used in this group was 16 to 30 mm. Upon stent dilation, the stricture returned almost to the maximum normal diameter of gastrointestinal dilation. Third, the duration of dilation was very long, with a typical period of stent placement for 3 to 7 days. Why was the therapeutic efficacy of temporary partially covered metallic stent dilation better than that of pneumatic dilation? We thought that this was mainly due to the stent expanding the strictured gastrointestinal region, causing chronic tearing of the strictured wall muscularis. As a stent gradually expanded with the body temperature of the patient, it took 12 to 24 h for a stent to reach 36 ℃. The stent thus expanded completely to reach the expected diameter. In our consideration, the wall muscularis was torn regularly by the metallic stent, and scars were relatively few when repaired. This resulted in a markably lower incidence of restenosis compared to that for pneumatic dilation.

Table 3 Strategies of intervention procedure for different benign strictures in upper gastrointestinal tract

Types of GIT stricture	Strategies
AS	TCSD > PD > PCSD > PUCSD
AS with fistula	PCSD > TCSD
New scar stricture	TCSD >PD > PCSD
Scar stricture	PCSD > TCSD > PD
Functional stricture (achalasia)	TCSD > PD >PCSD with antireflux

AS: anastomosis stricture, TCSD: temporary covered stent dilation, PD: pneumatic dilation, PCSD: permanent covered stent dilation, PUCSD: permanent uncovered stent dilation.

With different intervention procedures compared in consideration of the extents of lesion, incidences of complication, therapeutic efficacies, and degrees of acceptance of patients,

we found that partially covered metallic stents could provide excellent therapeutic effect. However, different strategies should be adopted to different types of lesion (Table 3). Development of biologically removable stents, which can be catabolized in 2 months after their placement, may provide a much longer retention time with no necessity for extraction[50-56].

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• CLINICAL RESEARCH •

Survivin expression induced by doxorubicin in cholangiocarcinoma

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Abstract

AIM: To study the role of survivin expression induced by chemotherapy agent (doxorubicin) in the development and anti-chemotherapy of cholangiocarcinoma.

METHODS: Expression of survivin was detected by SP immunohistochemical technique in 33 cases of cholangiocarcinoma, 28 cases of adjacent noncancerous bile duct, and 5 cases of benign bile duct lesions. Low concentration of doxorubicin (0.05 mg/l) was added in cultured cholangiocarcinoma cell line (QBC939). The expression of survivin was detected by RT-PCR and Western blot at 24 h and 48 h after adding doxorubicin.

RESULTS: Survivin was expressed in 24 of 33 cholangiocarcinoma cases (72.7%). In contrast, no expression of survivin in adjacent noncancerous and benign bile duct lesions was observed ($P<0.01$). No correlation was found between survivin expression and clinical features. Doxorubicin could markedly ($P<0.001$) up-regulate survivin mRNA and protein expression of QBC939 cells.

CONCLUSION: Overexpression of survivin in cholangiocarcinomas may play an important role in the development of cholangiocarcinoma, its relationship with prognosis of cholangiocarcinoma deserves further investigation. Higher expression of survivin is induced by doxorubicin in QBC939. Survivin expression may resist apoptosis induced by chemotherapy agents.

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INTRODUCTION

Survivin, a member of the inhibitors of apoptosis protein (IAP) family, is characterized by a unique structure that discriminates it from other members of the IAP family. It contains only a single BIR repeat and lacks a carboxy terminal RING finger domain. Survivin is expressed in the G_2/M phase of cell cycle in a cycle-regulated manner[1]. It directly binds to and inhibits both Caspase-3 and Caspase-7 activity, leading to arrest of apoptosis[2].

Survivin expression is not detectable in differentiated normal adult cells of any organ[3], but it is abundantly expressed in embryonic tissues and in a wide range of cancer tissues[4] including neuroblastoma[5], colorectal[6], stomach[7] and breast[8] carcinomas. It has been demonstrated recently that survivin is also frequently expressed in malignant pancreatic ductal tumors[9] and pancreatic adenocarcinoma[10]. Furthermore, the prognostic value of survivin expression has been reported in several human cancers[11].

Inducing apoptosis is the mechanism of chemotherapy agents killing tumor cells. But tumor cells resist chemotherapy agents because not only they overexpress MDR1/P-glycoprotein (P-gp) but also resist apoptosis induced by chemotherapy agents. Studies demonstrating resistance of survivin-transfected cells to anticancer drug-induced apoptosis[2] and sensitization to chemotherapy by survivin antisense treatment[12] have shown that survivin is implicated in sensitization to chemotherapy.

But in cholangiocarcinoma, survivin distribution and its implication for apoptosis inhibition are not clear at present. This study aimed to study the role of survivin expression induced by chemotherapy agent (doxorubicin) in the development and anti-chemotherapy of cholangiocarcinoma.

MATERIALS AND METHODS

Materials

Thirty-three specimens were obtained from patients with cholangiocarcinoma at the Department of General Surgery, Tongji Hospital of Tongji Medical College during the period from 1993 to 2001. There were 21 males and 12 females, and the mean age of the patients was 55.1 years (range from 34 to 79 years). The patients did not receive chemotherapy, radiation therapy or immunotherapy before surgery. Five specimens of benign bile duct lesions were also obtained. Formalin-fixed, paraffin-embedded blocks of tissue samples were taken from pathological archives. Serial sections of 4 μm were prepared from the cut surface of the blocks at the maximum cross-section of the tissue sample. Representative sections were stained with H&E in order to confirm the histopathological diagnosis. Human extrahepatic cholangiocarcinoma cell line QBC939 was established by Professor Wang SG (Third Military Medical University, China) and offered to us as a gift[13]. The cells were maintained as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 50 mL/L CO_2 at 37 ℃.

Methods

Immunohistochemical staining Immunohistochemical staining was carried out with the SP technique using the SP kit (Zhongshan Biotech Co., Beijing, China) after antigen retrieval by microwave pretreatment. Briefly, deparaffinized sections were immersed in a 0.1 M sodium citrate buffer (pH 6.0) and heated three times for 5 min each at a 15 min interval in a microwave oven at 600 W. After quenched in 3 % hydrogen peroxide and blocked, the sections were incubated with rabbit survivin polyclonal antibody (Neomarkers, USA; dilution 1:200) overnight at 4 ℃. Biotinylated antirabbit immunoglobulin and streptavidin conjugated to horseradish peroxidase were

subsequently applied. Finally, 3' , 3' -diaminobenzidine was used for color development, and hematoxylin was used for counterstaining. As a negative control, the sections were processed in the absence of primary antibody. Tissue sections from a hepatocellular carcinoma with a known strong expression of survivin were used as a positive control. A scoring method was used to quantitate the survivin expression in various samples examined. A mean percentage of positive tumor cells was determined in at least five areas at ×400 magnification. Patients with scores of less than 5% were defined as negative, otherwise they were defined as positive.

RT-PCR Low concentration of doxorubicin (0.05 mg/l, Pharmacia & Upjohn Co. Ltd.) was added in cultured cholangiocarcinoma cell line (QBC939). Expression of survivin was detected by RT-PCR before adding doxorubicin and at 24 h and 48 h after adding doxorubicin. Total RNA was prepared from subconfluent cultures with TRIzol reagent (Gibco, USA) according to the manufacture' s instructions. The primers were designed to amplify a fragment of survivin cDNA based on the reported sequence for human survivin. To normalize the amount of input RNA, RT-PCR was performed with primers for constitutively expressed β-actin gene. The survivin primers were 5' -CCCCATAGAGAACATAAA-3' (sense) and 5' -GGAATAAACCCTGGAAGTG-3' (antisense), giving rise to a 273 base pair polymerase chain reaction product. The β-actin primers were 5' -GTGCGTGACATTAAGGAG-3' (sense) and 5' -CTAAGTCATAGTCCGCCT- 3' (antisense), giving rise to a 520 base pair polymerase chain reaction product. The first strand cDNA synthesis and the subsequent PCR were performed with RNA PCR kit (AMV) using a programmed temperature control system set for 35 cycles, each consisting of denaturation at 94 ℃ for 45 s, annealing at 50 ℃ for 45 s, and extension at 72 ℃ for 45 s. Ten μL reaction mixture was electrophoresed on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining and quantified by an ImageQuant software. Survivin mRNA expression level was determined by survivin/β-actin protein.

Western blot Low concentration of doxorubicin (0.05 mg/l) was added in cultured cholangiocarcinoma cell line (QBC939). Expression of survivin was detected by Western blot before adding doxorubicin and at 24 h and 48 h after adding doxorubicin. Total cells were lysed with cell-lysis buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1% NP-40]. Twenty μg of protein was separated on 10% of SDS-PAGE gels and transferred to NC membranes. After blocked with 5% non-fat milk, the membranes were incubated with rabbit survivin polyclonal antibody (1:1 000 dilution) at 4 ℃ overnight. After washed three times the membranes were incubated with goat anti-rabbit IgG at room temperature for 1 hour. The signals were developed with the ECL kit (Amersham Pharmacia Biotechnology Inc.).

Statistical analysis

Association between survivin expression and various clinical and pathological variables was examined using χ^2 test or Fisher' s exact test. The data of PCR and Western blot were expressed as mean±SD. Student' s *t*-test was used for statistical analysis. $P<0.05$ was considered statistically significant.

RESULTS

Expression of survivin and associated clinicopathological variables

Survivin was prominently found in 24 of 33 cholangiocarcinoma cases (72.7%) by immunohistochemistry. Positive staining for survivin was located in the cytoplasm of tumor cells (Figure 1). In contrast, expression of survivin was observed neither in adjacent noncancerous bile ducts nor in benign bile duct lesions ($P<0.01$). No correlation was found between survivin expression and clinical features (Table 1).

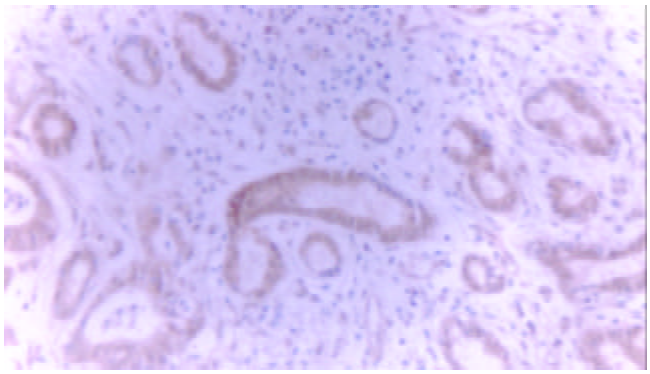

Figure 1 Expression of survivin in cholangiocarcinoma (×200, SP).

Table 1 Correlation between clinicopathological factors and survivin expression in cholangiocarcinoma

Clinical features	*n*	Survivin expression		χ^2	*P*
		n	%		
All patients	33	24	72.7		
Age (years)					
≤60	18	13	72.2	0.0051	0.9431
>60	15	11	73.5		
Sex					
Male	21	15	71.4	0.0491	0.8246
Female	12	9	75.0		
Tumor size (cm)					
≤2	13	10	76.9	0.1904	0.6626
>2	20	14	70.6		
Differentiation level					
High & Middle	29	21	72.4	0.0119	0.9133
Low	4	3	75.0		
Metastasis					
Positive	7	5	71.4	0.0076	0.9307
Negative	26	19	73.1		

Expression level of survivin mRNA

Doxorubicin could markedly ($P<0.001$) up-regulate survivin mRNA expression of QBC939 cells (Figure 2, Table 2).

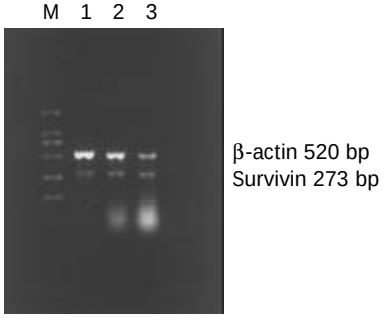

Figure 2 Expression of survivin in a human cholangiocarcinoma cell line. β-actin served as control. M: DL2 000 marker, 1: Normal Qbc939, 2: 24 h after adding doxorubicin, 3: 48 h after adding doxorubicin.

Table 2 Expression level of survivin mRNA

Group	*n*	Survivin / β-actin	*t*	*P*
A	7	0.4210±0.0551	^{b}t=45.89, ^{d}t=12.54	^{b}P, ^{d}P<0.001
B	7	0.8481±0.0713	^{b}t=26.11	^{b}P<0.001
C	7	1.7034±0.0493		

Survivin mRNA expression level was determined by survivin / β-actin protein. Data were expressed as mean±SD, b *vs* C (48 h after adding doxorubicin), d *vs* B (24 h after adding doxorubicin), A: Normal Qbc939.

Expression level of survivin protein

Doxorubicin could markedly (P<0.001) up-regulate survivin protein expression of QBC939 cells (Figure 3, Table 3).

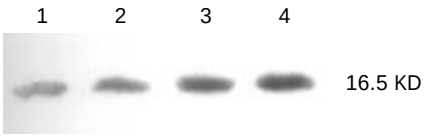

Figure 3 Expression of survivin in a human cholangiocarcinoma cell line. 1 and 2: Normal Qbc939, 3: 24 h after adding doxorubicin, 4: 48 h after adding doxorubicin.

Table 3 Expression level of survivin protein

Group	*n*	OD value	*t*	*P*
A	6	204.568±1.387	^{b}t=17.99, ^{d}t=11.23	^{b}P, ^{d}P<0.001
B	6	311.105±1.539	^{b}t=11.02	^{b}P<0.001
C	6	339.989±1.872		

Data were expressed as mean±SD, b *vs* C (48 h after adding doxorubicin), d *vs* B (24 h after adding doxorubicin), A: Normal Qbc939.

DISCUSSION

Expression of survivin was detected in 72.7% of cholangiocarcinomas. In contrast, no expression of survivin in adjacent noncancerous and benign bile duct lesions was observed (P<0.01). Using a similar polyclonal antibody, survivin expression was detected in 93% of malignant melanomas[14], 81% of basal cell carcinomas, 92% of cutaneous squamous cell carcinomas[15], 70% of hepatocellular carcinomas[16], 88% of gastric carcinomas[17], 100% of oesophageal cancers[18], 88% of pancreatic adenocarcinomas[10] and 74% of ovarian carcinomas[19]. Our study demonstrated a high expression of survivin in cholangiocarcinoma as in other human malignancies.

There was no correlation between survivin expression and any clinical or pathological characteristics of cholangiocarcinoma. A similar absence of correlation was also noted in previous observations including gastric[7,20], colorectal[6,21] and breast cancers[8]. Though many reports have shown that survivin was an independent prognostic factor for various cancers[19,22-27], such a distribution of clinicopathological features and the high prevalence of survivin expression in cholangiocarcinoma might have rendered the power of this study insufficient to demonstrate any correlation between survivin expression and any clinical or pathological characteristics. Thus, the relationship with prognosis deserves further investigation.

Substantial evidences have also shown that during chemotherapy, changes in expression levels of survivin might provide information about chemo-sensitivity or chemo-resistance of tumors[28,29]. In the present study, low concentration of chemotherapy agent doxorubicin (0.05 mg/l) could markedly (P<0.001) up-regulate survivin mRNA and protein expression of QBC939 cells. These results suggested a direct link between survivin expression and bile duct carcinoma cell susceptibility to doxorubicin. That is, higher survivin expression may directly down-regulate chemo-sensitivity. It may be one of the mechanisms of anti-chemotherapy in cholangiocarcinoma.

In summary, a high expression of survivin in cholangiocarcinoma may play an important role in the development of cholangiocarcinoma, the relationship with prognosis deserves further investigation. Higher expression of survivin could be induced by doxorubicin in QBC939 and might resist apoptosis induced by chemotherapy agents. These results provide several exciting therapeutic possibilities. Initial evidence *in vitro* and *in vivo* has shown that targeting survivin may provide a viable approach to kill cancer cells selectively[30,31]. Inhibition of survivin expression by molecular manipulation has been reported to improve the effectiveness of chemotherapy[12,31-33] and produce impact on radiation therapy[34-37].

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• BRIEF REPORTS •

Effects of gastric pacing on gastric emptying and plasma motilin

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Abstract

AIM: To investigate the effects of gastric pacing on gastric emptying and plasma motilin level in a canine model of gastric motility disorders and the correlation between gastric emptying and plasma motilin level.

METHODS: Ten healthy Mongrel dogs were divided into: experimental group of six dogs and control group of four dogs. A model of gastric motility disorders was established in the experimental group undergone truncal vagotomy combined with injection of glucagon. Gastric half-emptying time ($GEt_{1/2}$) was monitored with single photon emission computerized tomography (SPECT), and the half-solid test meal was labeled with an isotope-^{99m}Tc sulfur colloid. Plasma motilin concentration was measured with radioimmunoassay (RIA) kit. Surface gastric pacing at 1.1-1.2 times the intrinsic slow-wave frequency and a superimposed series of high frequency pulses (10-30 Hz) was performed for 45 min daily for a month in conscious dogs.

RESULTS: After surgery, $GEt_{1/2}$ in dogs undergone truncal vagotomy was increased significantly from 56.35±2.99 min to 79.42±1.91 min ($P<0.001$), but surface gastric pacing markedly accelerated gastric emptying and significantly decreased $GEt_{1/2}$ to 64.94±1.75 min ($P<0.001$) in animals undergone vagotomy. There was a significant increase of plasma level of motilin at the phase of IMCIII (interdigestive myoelectrical complex, IMCIII) in the dogs undergone bilateral truncal vagotomy (baseline *vs* vagotomy, 184.29±9.81 pg/ml *vs* 242.09±17.22 pg/ml; $P<0.01$). But plasma motilin concentration (212.55±11.20 pg/ml; $P<0.02$) was decreased significantly after a long-term treatment with gastric pacing. Before gastric pacing, $GEt_{1/2}$ and plasma motilin concentration of the dogs undergone vagotomy showed a positive correlation ($r=0.867$, $P<0.01$), but after a long-term gastric pacing, $GEt_{1/2}$ and motilin level showed a negative correlation ($r=-0.733$, $P<0.04$).

CONCLUSION: Surface gastric pacing with optimal pacing parameters can improve gastric emptying parameters and significantly accelerate gastric emptying and can resume or alter motor function in a canine model of motility disorders. Gastric emptying is correlated well with plasma motilin level before and after pacing, which suggests that motilin can modulate the mechanism of gastric pacing by altering gastric motility.

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INTRODUCTION

The incidence of gastric motility disorder has been increasing, but the mechanisms of the development of this disease remain obscure, and specific and effective therapy are lacking. The current treatment mainly relies on conventional prokinetic agents. However, some patients with motility disorder can not undergo a chronic treatment with prokinetic drugs because of their side effects[1-3]. In addition, tachyphylaxis may occur sooner or later and refractoriness to prokinetic agents is observed in a large number of patients. The limited efficacy of drugs in gastric motility disorder has led investigators to examine the effect of gastric pacing on gastric emptying. In the wake of cardiac pacing ,some investigators hope that gastric pacing would be a new way to treat refractory gastric motility disorder. The role of gastric pacing with application of implanted gastric serosa electrodes, suction intraluminal electrodes or gastric mucosal electrodes in gastrointestinal (GI) tract has been greatly discussed in western countries[4-23]. Most studies were restricted to animal experiments because of the invasiveness, substantial risk, and complications associated with the surgical procedure. Howerer, the study of surface gastric pacing was rarely reported in medical literature. To determine the therapeutic efficacy of surface gastric pacing, we combined bilateral truncal vagotomy with administration of glucagon to establish a canine model of gastric motility disorder. Then, we observed the effects of surface gastric pacing on gastric emptying and plasma motilin level before and after pacing, and also explored the possible mechanism of gastric pacing.

MATERIALS AND METHODS

Animals and surgical preparation

Ten healthy Mongrel dogs were divided into: experimental group of six dogs (four males and two females) 12.5-17.5 (average 14.5) kg and control group of four dogs (two males and two females) 13.0-17.0 (average 15.5) kg. Bilateral truncal vagotomy was performed in the experimental group under aseptic conditions with intravenous anaesthesia of pentobarbital sodium after an overnight fasting. The initial dosage of the anesthetics was 30 mg/kg,which was supplemented with 3 mg/kg if necessary on the basis of corneal reflex. Artificial ventilation was given during surgery. After surgery, the animal was first transferred to an intensive care cage for a few hours and then to the regular cage after a complete recovery from anesthesia. The protocal was approved by the Institution of Animal Care of the Third Military Medical University.

Experimental procedure

The dogs were allowed to recover completely from surgery for 2 to 3 wk before any study. The study was conducted in

three sessions for each dog and experiments were performed on all the dogs in awake state. The dogs stood quietly on a table in a canvas support sling when gastric pacing was administered and gastric emptying was assessed. In the first session, we established the basic characteristics for gastric emptying in both control dogs and experimental dogs. In the second session, an intravenous injection of glucagon (500 μg/kg)[4,24] was administered. Gastric emptying of semi-solid food was monitored in all 10 dogs in the first 5 min after glucagon injection. Three days later, a second experiment with glucagon was performed, and we utilized gastic pacing to observe if the effects on drug could be altered, and gastric emptying was monitored for 2 h with gastric pacing. In the third session, gastric pacing was performed for 45 min after meal daily for a month in dogs of the experimental group to observe the chronic efficacy of a long-term treatment with gastric pacing, and gastric emptying was assessed for 2 h after cessation of gastric pacing.

Gastric pacing

In order to simulate the basic electrical rhythm (BER) and induce release of acetylcholine from intramural cholinergic fibers, gastric pacing stimulus consisted of two signals: a continuous similar-sine basic wave with a low-frequency (1.1-1.2 times the intrinsic slow-wave frequency) and a superimposed series of high frequency pulses (10-30 Hz), the number of a series of pluse was 15-40/cycle. The pacing signals from a pacemaker were delivered with a pair of bipolar skin electrodes. Stimulating electrodes were placed on commonly used positions of the abdominal region (localization through X ray). A long-term surface gastric pacing was performed for 45 min after meal daily for a month.

Measurement of gastric emptying

Gastric emptying was monitored with single photon emission computerized tomography (SPECT) (GE Inc.,USA). The semi-solid test meal consisted of 90 mg of commercial black sesame powder labeled with 74 MBq of ^{99m}Tc-sulfur colloid and 120 ml water. The study of gastric emptying consisted of two procedures, with or without gastric pacing, in a randomized order. On the day of gastric emptying test, the animal was fasted over 16 h. Immediately after the intake of isotope-labeled semi-solid meal, the maximum count rates were determined. Afer a first image was taken, the other sets were taken at an interval of 15 min for 2 h. The gastric region of interest was outlined by hand on the initial image by a nuclear medicine technologist who had no interest in the outcome of the study. To reduce the error resulted from the physical radioactive decay of isotope ^{99m}Tc, we corrected and normalized the percentage of semi-solid food retained in the stomach over time and chose the linear-fitting decay program to extrapolate $GEt_{1/2}$.

Measurement of plasma concentration of motilin

Plasma level of motilin was determined with commercially available RIA (radioimmunoassay) kits. Blood was collected before and after vagotomy and after a long-term treatment of gastric pacing at the start of phase III of interdigestive myoelectric complex (IMCIII) respectively. Samples of peripheral venous blood were collected in lyophilized tubes containing 40 μl of 0.3 mol/L Na_2-EDTA and 2 000 u trasylol. Immediately, plasma was separated with centrifugation at 4 000 r/min at 4 ℃. The plasma should be frozen within 2 hours and then stored at -70 ℃ for batch assay with a well-established motilin immunoassay[20] (The motilin kit was supplied by the Radioimmunologic Center of PLA General Hospital, China).

Statistical analysis

Analysis of variance and paired Student' s *t* test and linear correlation analysis (SPSS 10.0 software) were performed to investigate the effects of gastric pacing on gastric emptying and plasma motilin level in dogs of the two groups. Statistical significance was assigned for $P<0.05$. All data were presented as means±SD.

RESULTS

Basic condition

All dogs generally tolerated well the surgical procedure and surface gastric pacing. Large doses of glucagon injected intravenously triggered hyperglycemia in the diabetic setting and plasma glucose levels were increased sharply from 81-114 mg/dl to 200-233 mg/dl. Only two of the ten dogs were sensitively induced by glucagon to show nausea and vomiting, but the adverse symptoms rapidly disappeared and did not lead to any complication. Long-term gastric pacing resulted in a significant weight gain. The average weight (11.3±2.4 kg) after surgery was significantly lower than that before surgery (15.5±3.2 kg, $P<0.01$), but after a long-term treatment with gastric pacing weight gain was found to be 14.7±2.7 kg ($P<0.05$) in dogs of the experimental group.

Effects of vagotomy and glucagon on gastric emptying

Gastric emptying data were collected from each dog in all conditions.Results are summarized in Table 1 and Figure 1. The data before and after vagotomy were compared, and $GEt_{1/2}$ of the dogs undergone truncal vagotomy was significantly increased from 56.35±2.99 min to 79.42±1.91 min ($P<0.001$). To observe the effects of glucagon alone on gastric emptying, we compared the $GEt_{1/2}$ of control dogs and the dogs undergone vagotomy before and after glucagon was administered (no gastric pacing was applied). In comparison with that before injection of glucagon, the $GEt_{1/2}$ of the dogs undergone vagotomy was significantly longer after glucagon was administered (108.24±10.75 min, $P<0.01$). However, the delay of gastric emptying in control dogs after injection of glucagon was not statistically significant (57.73±1.65 min *vs* 56.15±2.34 min, $P>0.05$).

Table 1 Effects of gastric pacing on gastric emptying parameters

Groups	*n*	$GEt_{1/2}$ (min)		
		No pacing	Acute pacing	Long-term pacing
Control	4	56.15±2.34	58.52±4.77	
Control + glucagon	4	57.73±1.65	56.90±2.53	
Vagotomy	6	79.42±1.91[a]	74.41±6.10	64.94±1.75[d]
Vagotomy+ glucagon	6	108.24±10.75[b]	76.93±8.55[c]	

All the data were expressed as means ± SD. [a]$P<0.001$ *vs* control, [b]$P<0.01$ *vs* before injection of glucagon, [c]$P<0.05$ *vs* before acute pacing, [d]$P<0.001$ *vs* before a long-term pacing.

Effects of acute gastric pacing on gastric emptying

To investigate the acute effects of gastric pacing on gastric emptying in dogs undergone truncal vagotomy in combination with injection of glucagon and in control dogs with injection of glucagon, we observed gastric emptying for 2 h during gastric pacing. The $GEt_{1/2}$ of the dogs undergone vagotomy and injection of glucagon was significantly shorter with pacing than that without pacing (108.24±10.75 min *vs* 76.93±8.55 min, $P<0.01$). However, such an acute pacing had no significant effect on the $GEt_{1/2}$ of control dogs with injection of glucagon (57.73±1.65 min *vs* 56.90±2.53 min, $P=0.4$) or when only

vagotomy was performed (79.42±1.91 min *vs* 74.41±6.10 min, P=0.3).

Effects of chronic gastric pacing on gastric emptying

To investigate the effects of chronic gastric pacing on gastric emptying, we compared the $GEt_{1/2}$ before pacing with that after a long-term gastric pacing of 30 d in the dogs undergone vagotomy. Gastric pacing significantly accelerated gastric emptying in animals undergone vagotomy (79.42±1.91 min *vs* 64.94±1.75 min, P<0.001) and significantly decreased retention of gastric isotope (Figure 1).

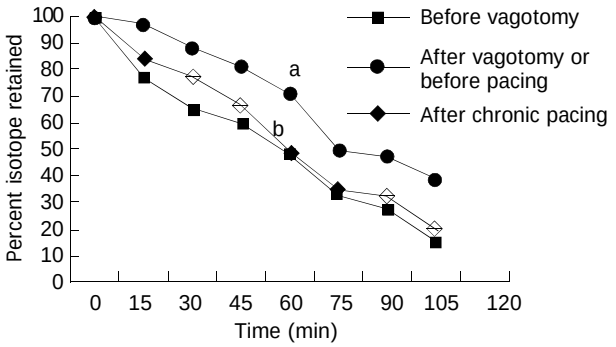

Figure 1 Effects of vagotomy and gastric pacing on gastric isotope retention during monitoring for 120 min. Data were expressed as means ± SD, n=6. [a]P<0.005 *vs* before vagotomy, [b]P<0.005 *vs* before pacing (by analysis of variance at 120 min).

Effects of gastric pacing on plasma gastric motilin

There was a significant increase of plasma motilin concentration in the phase of IMCIII after bilateral truncal vagotomy (baseline *vs* vagotomy, 242.09±17.22 pg/ml *vs* 184.29±9.81 pg/ml; P<0.01). But plasma motilin concentration was significantly lower after a long-term surface gastric pacing than that before gastric pacing (212.55±11.20 pg/ml; P<0.02).

Correlation of plasma gastric motilin and gastric emptying

Statistic analysis indicated that $GEt_{1/2}$ (79.42±1.91 min) and plasma motilin concentration (242.09±17.22 pg/ml) in the phase of IMCIII of the dogs undergone vagotomy had a positive correlation (r= 0.867, P<0.01) before surface gastric pacing, that is, vagotomy evoked the increase of plasma motilin level which clearly disrupted gastric emptying. But after a long-term gastric pacing, $GEt_{1/2}$ (64.94±1.75 min) and plasma motilin level (212.55±11.20 pg/ml) had a negative correlation (r=-0.733, P<0.04), that is, plasma motilin release was inclined to restore its normal level, which might be helpful to improve gastric emptying.

DISCUSSION

We used bilateral truncal vagotomy in combination administration of glucagon to establish a canine model of gastric motility disorders, which can simulate some aspects of diabetic gastroparesis. Our study demonstrated that suface gastric pacing at 1.1-1.2 times the intrinsic slow-wave frequency superimposed series of high frequency pulses (10-30 Hz) was able to entrain propagated slow waves to replace the spontaneous ones and to improve gastric emptying in dogs after vagotomy and injection of glucagon. It is considered that gastric pacing could be proven to be an effective therapeutic approach for the treatment of gastric motility disorders[13,20-22,25-27].

Complete abdominal vagotomy has been shown to induce gastric stasis and delay emptying of solid food in both humans and animals[4,11]. It could change the contractile pattern during interdigestive migrating motor complex III (IMMCIII)[28]. Extrinsic denervation of the antrum could diminish the frequency of antral action potentials and retard gastric emptying of solids. Our study indicated that gastric emptying of semi-solid food was obviously impaired after vagotomy in six dogs and confirmed the finding of previous researchers[4,11] despite difference in their techniques of measuring gastric emptying. Previous studies have demonstrated that glucagon could delay gastric emptying of intact dogs[29], but our study did not find that glucagon was able to delay gastric emptying in the healthy dogs, suggesting that the dogs undergone vagotomy were more susceptible to gastric motility disorder. The mechanisms by which glucagon delayed gastric emptying in both humans and dogs have not been fully understood. This delay might associate with acute hyperglycemia-evoked gastric slow wave dysrhythmias by endogenous prostaglandins[24,30], such as tachygastria, tachyarrhythmia, bradyarrhythmia, asystolia (electrical silence), and gastric fibrillation which is a complete disorganization of gastric electrical activity due to impairment of coupling and propagation of gastric slow waves causing unpropagated antral contractions and exacerbating the hyperglycemia-evoked antral hypomotility[31-33]. In addition, hyperglycemia might have inhibitory effects on the spike potentials that could induce antral contractions or possible indirect effects of glucagon on gastric emptying through the release of hormones or neurotransmitters[4].

At present, controversial findings were reported about the effects of gastric pacing on gastric emptying. McCallum *et al*.[13,20,22] showed that there was an increase in gastric emptying. On the other hand, Eagon *et al*.[6] observed that neither the motility index nor gastric emptying rate was consistently changed by stimulation at any frequency. In our study, we employed a 1.1-1.2 times the intrinsic physiologic slow-wave frequency superimposed series of high frequency pulses (10-30 Hz) in surface gastric pacing which resulted in a significant acceleration of gastric emptying of semi-solid food in dogs undergone vagotomy but not in intact dogs. Our findings were consistent with those of Bellahsene *et al*.[4] who employed a frequency similar to or slightly higher than the intrinsic slow-wave frequency.

Our study also evaluated the effects of total abdominal vagotomy and surface gastric pacing on plasma concentration of motilin in the phase of IMCIII. Motilin is a straight chain peptide containing 22 amino acids and has a molecular weight of approximately 2700. It has been found that plasma motilin levels varied in a cyclic fashion during the fasting state in accordance with the interdigestive migrating motor complex (IMMC), and IMMC III was concurrent with the peak of plasma motilin level[34]. Motilin has been known to induce IMMC III contractions through cholinergic neural pathways, 5-hydroxytryptamine (HT) receptors, and alpha receptors[35,36]. IMMC can clean large indigestible food residues, enhance antroduodenal and gallbladder coordination, stimulate pancreatic and bile secretion, and keep the small intestine free from stasis[37]. Our results showed that there was a significant difference in plasma motilin concentration in the phase of IMCIII before and after the operation and after gastric pacing. The significant increase of plasma motilin level in the dogs after truncal vagotomy might be explained that total abdominal vagotomy or a complete extrinsic vagal gastric denervation could markedly delay gastric empty and induce gastric stasis, and reduce the sensitivity of motilin receptors, which might feedbackly lead to the release of plasma motilin. After a long-term surface gastric pacing, the delay of gastric emptying was improved and plasma motilin level gradually restored normal. At prsent, its mechanism is not clearly determined. In addition, our study showed gastric emptying was correlated well with plasma motilin level in the phase of IMCIII before and after gastric pacing, which implies that there is a possibility for

motilin to mediate the process of gastric pacing by altering gastric motility.

It has been found that gastrointestinal motility is regulated by a spatio-temporally-coordinated electrical pattern called gastrointestinal myoelectrical activity (GMA) that is paced by the GI pacemaker[38]. Recently, it has been recognized that interstitial cells of Cajal known as pacemaker cells could generate rhythmically electrical pacemaker activities (or electrical slow waves) which regulate the frequency of contractions of the tunica muscularis for GI motility[39-44]. The normal patterns of GMA present temporal evolution from endogenous rhythmic oscillation to bursting of spikes associated with contractions, and also order spatial propagation of the oscillating waves. Spatio-temporal modeling of the GMA has been established, thanks to the advances in electronic technology. Therefore, we employed a continuous similar-sine basic wave with a low- frequency (10%-20% high than intrinsic gastric frequency) superimposed a series of high frequency pulses (10-30 Hz) as signals of surface gastric electrical pacing. In contrast to gastric pacing through implanted serosal electrodes and the suction intraluminal electrodes in gastrointestinal tract, surface gastric pacing is more simple and noninvasive. In addition, surface gastric pacing does not require any surgical procedures such as laparotomy or laparoscopy. So this technique is a very attractive, reliable and acceptable candidate method to treat gastric motility disorders.

Although gastric pacing could restore a normal pattern of slow wave and an efficient gastric motor function and improve gastric emptying, its mechanism remains unclear[45]. Recently, Qian *et al.*[15] reported gastric pacing at a frequency similar to or just above that of the native slow wave to induce gastric slow wave was not via the cholinergic nerves, because although atropine was used to block the vagal activity, gastric pacing could still entrain and normalize the irregular rhythm of gastric slow wave. Moreover, spectral analysis of heart rate indicated that gastric pacing had no effect on the extrinsic autonomic functions. Accordingly, we presume that the mechanism of gastric pacing at a frequency slightly higher than the intrinsic frequency to induce gastric slow wave may be via some other neurohumoral pathways or directly leads to local activation of interstitial cells of Cajal and/or smooth muscle cells. Gastric electrical stimulation with a frequency much higher (about 10-1 200 cycles/min) than that of the intrinsic slow wave probably could act on sensory fibers directed to the central nervous system[46]. Gastric electrical stimulation with a frequency 10-100 Hz might be possibly associated with autonomic and enteric nervous system changes, likely induce the release of acetylcholine from the intramural cholinergic fibers, which in turn stimulated the muscular cell contraction, because its effect was prevented by a previous administration of atropine[47-49]. In addition, gastrointestinal hormones such as motilin or NANC neurotransmitter VIP might also mediate the process of gastric pacing[47,50].

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• BRIEF REPORTS •

Surgical salvage therapy of anal canal cancer

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Abstract

AIM: To evaluate the results of salvage resection in the management of persistent or locally recurrent anal canal cancer.

METHODS: Details of all patients with anal canal cancer treated from 1978 to 1994 at Cancer Hospital of Chinese Academy of Medical Sciences (CAMS) were reviewed retrospectively. Sixteen patients who presented with persistent or locally recurrent anal canal cancer received salvage surgery. Before surgery all of the patients had received radiotherapy alone as their primary treatments.

RESULTS: Of the 16 patients, 14 received salvage abdominoperineal resection (APR) and two had transanal local excision. There were no deaths attributable to operation. Delayed healing of the perineal wound occurred in eight patients. Complications unrelated to the perineal wound were found in five patients. The median follow-up time was 120 (range 5-245) months after salvage surgery. Nine patients died of disease progression, with a median survival time of 16 (range 5-27) months. Six patients had a long-term survival.

CONCLUSION: Salvage resection after radiotherapy can yield a long-time survival in selected patients with anal canal cancer. However it offers little hope to patients with T_4 and/or N_{2-3} tumors.

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INTRODUCTION

Anal canal cancer is rare and only accounts for 2% to 4% of all anorectal neoplasms[1,2]. Radiotherapy alone or concomitant chemoradiotherapy currently is considered as a standard treatment for most of the patients[3,4]. Although disease control was reported to be excellent, as many as 33% of patients wouldl develop locoregional disease progression[3-5]. Because this disease is uncommon, there has been no randomized study that compares different salvage approaches[6]. The aim of this study was to review our experience in salvage surgery for patients who developed local disease recurrence after radiotherapy.

MATERIALS AND METHODS

Materials

From 1978 to 1994, 83 patients with biopsy-proven anal canal cancer were treated with curative intent at Cancer Hospital of Chinese Academy of Medical Sciences (CAMS). Primary radiotherapy failed to produce any regression of the primary tumor in 16 patients. These 16 patients, including eleven who presented with a persistent disease and five patients who had a recurrent disease, were treated with salvage surgery. Primary tumors were staged in accordance with the criteria of the American Joint Committee on Cancer (AJCC)[7]. Diagnosis of lymph node metastasis was made clinically, with radiological investigations such as computerized tomography scanning, and confirmed histologically. Pretreatment characteristics of these patients are listed in Table 1.

Table 1 Pretreatment characteristics (n=16)

Characteristics	No. of patients (n)
Demographics	
Median age(y)(range)	56(45-70)
Males / females	9 / 7
Histology	
Squamous	15
Basaloid	1
Clinical stage (UICC TNM)	
T_1	1
T_2	15
T_3	2
T_4	1
N_0	7
N_2	6
N_3	3
Stage II	7
Stage IIIB	9

Treatment

Of the 16 patients, 15 received external beam radiation therapy (EBRT) alone. The mean dose of EBRT was 40 Gy(range 30-75 Gy). One patient was implanted with ^{192}Ir sources after EBRT. The brachytherapy dose was 14 Gy. No patients received adjuvant or concomitant chemotherapy. After completion of primary treatment, the patients were followed up according to a standard protocol[8].

RESULTS

Surgical results

Among the 16 patients who failed initial therapy, 14 underwent salvage abdominoperineal resection (APR) and two had transanal local excision. Partial prostatic resection was performed on two males. There were no deaths attributable to operation. Fourteen patients had their perineal wounds packed open for hip bath. Healing time of the perineal wound

exceeding 3 months was considered to be delayed, which occurred in eight patients. In four patients the healing time of perineal wound exceeded six months and in one patient the healing was not achieved within two years. Other complications unrelated to perineal wound were recorded in five patients. These included one small bowel obstruction treated conservatively, one perineourethra fistula after prostatic resection, one abdominal wound infection and two neurocystitis.

Oncological results

All the patients were followed up. The median follow-up time was 120 (range 5-245) months after salvage surgery. At the time of the last follow-up nine patients died of disease progression, with a mean survival time of 16(range 5-27) months. In these patients, eight presented with a persistent disease and one had a recurrent disease. The median follow-up time among survivors was 173 (range 98-245) months. Six patients survived for more than 10 years. According to the initial tumor stage, one of seven patients with $T_2N_0M_0$ died 18 months after resection whereas eight of nine patients with $T_{1-4}N_{2-3}M_0$ disease were not controlled. Of the 16 patients, only one patient presented with a second locoregional disease recurrence and was salvaged with radiotherapy.

DISCUSSION

Despite progresses in chemoradiotherapy of anal epidermoid cancer[3,4,9,10], a substantial percentage of patients, particularly those with more advanced disease, still developed a local failure and demand salvage therapy[11,12]. Surgical resection was often recommended as the most appropriate salvage method[13-15]. The overall survival rate for surgical salvage at 5 years was about 30-60%[16,17]. Some investigators reported disappointingly low success rates[18] and alternative approaches have been suggested, such as low dose radiotherapy or combined chemoradiotherapy[19]. In the present study, six of the 16 patients who underwent appropriate surgical treatment survived for more than 10 years. These results are similar to those of Longo *et al*[20], who reported a survival rate of 53% among 17 patients and Ellenhorn *et al*[21], who noted that 44% of 38 patients survived for 5 years after salvage resection.

It is well known that delayed healing of perineal wounds is a common complication for patients after radiotherapy. Earlier investigators reported the perineal wound complication rate was up to 30%[16,17,21]. In the study by Nilsson *et al*[22], the rate of delayed healing was 66 % (22 of 35). No difference was detected in the radiation dose delivered between patients with delayed healing and those in whom healing was achieved within 3 months. Also in rectal cancer, preoperative radiotherapy whether given in 5.0-Gy fractions or using conventional 1.8-2.0-Gy fractions, always made the healing of perineal wounds delay[23]. In 14 patients who received salvage APR, eight presented with a delayed perineal wound healing and one did not have a wound healing within two years. It might help the healing of large defects created in an irradiated field by using an omentoplasty or musculo-cutaneous flap[24,25].

Of the five patients who presented with local recurrence after having achieved complete disease remission, three survived for more than 10 years. In contrast, among the 11 patients who presented with persistent diseases, eight died of the diseases within 3 years. There was a better 10-year survival among patients with recurrences compared with that in patients with a persistent disease. These findings are consistent with those of Nilssio *et al*[22], who reported a significantly better 5-year survival among patients with recurrences (82% *versus* 33%) and Allal *et al*[17](56% *versus* 23%), but contradicted with those of Pocard *et al*[16], who reported that patients with a persistent disease had a longer survival time (74 *versus* 25 months). The difference may be related to the tumors' stage and nodal status at initial presentation, or, alternatively, may reflect more aggressive biologic phenotypes of tumors that are different in response to radiotherapy.

The effect of salvage surgery appeared to correlate with the initial disease stage[26-28]. In the study by Allal *et al*[17], 41% of patients who failed local treatment of $T_{2-3}N_0M_0$ anal canal cancer were successfully salvaged, compared with 17% of patients with $T_{1-4}N_{2-3}M_0$ anal canal cancer. The effects of initial lymph node involvement and tumor extent on patients' outcome after surgical salvage therapy also were stressed by Ellenhorn *et al*[21]. In the present study, eight of 9 patients who died of the disease presented with $T_{1-4}N_{2-3}M_0$ anal canal cancer, whereas most of the survivors had a $T_2N_0M_0$ anal canal cancer.

In summary, salvage APR after radiotherapy has a high complication rate, but can bring a long-time survival in selected patients with anal canal cancer. Since patients with T_4 and/or N_{2-3} tumors could not obtain much benefit from salvage surgery, salvage chemoradiotherapy needs to be further investigated.

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• BRIEF REPORTS •

Utility of serum CA19-9 in diagnosis of cholangiocarcinoma: In comparison with CEA

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Abstract

AIM: The diagnosis of cholangiocarcinoma is often difficult, making management approaches problematic. A reliable serum marker for cholangiocarcinoma would be a useful diagnostic test. The aims of our study were to evaluate the usefulness of a serum CA19-9 determination in the diagnosis of cholangiocarcinoma.

METHODS: We prospectively measured serum CA19-9 and CEA concentrations in patients with cholangiocarcinoma (n=35), benign biliary diseases (n=92), and healthy individuals (n=15). Serum CA19-9 and CEA concentrations were measured by an immunoradiometric assay without knowledge of the clinical diagnosis.

RESULTS: The sensitivity of a CA19-9 value >37 KU·L^{-1} and a CEA value >22 μg·L^{-1} in diagnosing cholangiocarcinoma were 77.14% and 68.57%, respectively. When compared with the benign biliary diseases group, the true negative rates of serum CA19-9 and CEA were 84.78% and 81.52%, respectively. The false positive rates of serum CA19-9 and CEA were 15.22% and 18.48%, whereas the accuracy of serum CA19-9 and CEA were 82.68% and 77.95%, respectively. Serum CA19-9 and CEA concentrations were significantly elevated (P<0.001 and P<0.05) in patients with cholangiocarcinoma (290.31±5.34 KU·L^{-1} and 36.46±18.03 μg·L^{-1}) compared with patients with benign biliary diseases (13.38±2.59 KU·L^{-1} and 13.84±3.85 μg·L^{-1}) and healthy individuals (12.78±3.69 KU·L^{-1} and 11.48±3.37 μg·L^{-1}). In 15 patients undergoing curative resection of cholangiocarcinoma, the mean serum CA19-9 concentration was decreased from a preoperative level of 286.41±4.36 KU·L^{-1} to a postoperative level of 62.01±17.43 KU·L^{-1} (P<0.001), and the mean serum CEA concentration from 39.41±24.35 μg·L^{-1} to 28.69±11.03 μg·L^{-1}(P<0.05). In patients with cholangiocarcinoma, however, no correlation was found between serum CEA and CA19-9 concentrations (r=0.036).

CONCLUSION: These data suggest that the serum CA19-9 determination is a useful addition to the available tests for the differential diagnosis of cholangiocarcinoma. Serum CA19-9 is an effective tumor marker in diagnosing cholangiocarcinoma, deciding whether the tumor has been radically resected and monitoring effect of treatment.

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INTRODUCTION

Cholangiocarcinoma is a malignant tumor arising from bile duct epithelium. Unlike most human cancers, a pathological diagnosis of cholangiocarcinoma is often extremely difficult because of its location, size, and desmoplastic characteristics[1-3]. Percutaneous fine needle aspiration is frequently not possible because many of these tumors are located in the liver hilum amid large vascular structures[4,5]. Furthermore, tumor masses are often not even identifiable by CT, ultrasound, or magnetic resonance imaging[6-8]. Endoscopic approaches are also of limited usefulness in tissue diagnosis because of the desmoplastic nature of these cancers. Indeed, bile cytology obtained at endoscopic retrograde cholangiography has a sensitivity of only 33-56%[1,9-11], endobiliary brush cytology of 50-68%[12-14], and endoscopic transpapillary biopsy of 53-86% for detecting cholangiocarcinoma[15-18]. Because of the problems in obtaining diagnostic tissues, treatment and management decisions for patients with biliary disease that may be malignant are problematic.

Of the two possible tumor markers available for detecting cholangiocarcinomas, carcinoembryonic antigen (CEA) is a glycoprotein tumor marker with the immunodeterminant present on the protein moiety of the molecule. The other, carbohydrate antigen 19-9 (CA19-9), is a mucin-type glycoprotein in serum with the immunodeterminant present on the carbohydrate moiety of the molecule. Both tumor markers have been investigated for the diagnosis of malignancies in the stomach, colon and pancreas[19-23] but have not been gained widespread use in bile duct. It has been reported that the sensitivity and specificity for CA19-9 value >37 KU·L^{-1} for cholangiocarcinoma with primary sclerosing cholangitis (PSC) were 60% to 93% and 78% to 98%, respectively[9,16,21,24-27]. The corresponding indexes of CEA value >22 μg·L^{-1} were 53% to 84% and 50% to 79%[25-30]. Although widely used as a tumor marker, the clinical value of serum CA19-9 determination in the diagnosis of cholangiocarcinoma in the absence of PSC is unknown. Thus, the objective of this study was to determine the clinical usefulness of CA19-9 value for cholangiocarcinoma.

MATERIALS AND METHODS

Patients

From January 1995 to February 2003, we prospectively obtained serum samples from patients undergoing evaluation for benign and malignant biliary disease at the First Hospital of Xi' an Jiaotong University and Luoyang Central Hospital. Serum samples were also prospectively obtained from healthy individuals who served as the disease control group. Patients with the diagnosis of PSC were excluded from this study. Clinical information was obtained by a thorough review of

the medical histories. This study included 35 patients with cholangiocarcinoma, 92 patients with benign biliary diseases, and 15 healthy individuals. Of the 35 patients with cholangiocarcinoma, the diagnosis was established by surgical biopsy in 25 patients, endoscopic biopsy and brushing in six patients, and fine needle aspiration in four patients. In patients with cholangiocarcinoma, the stage and resectability of the tumors were ascertained using information obtained by imaging studies or at the time of surgery. Unresectability was defined by Bismuth stage 4 cancer arising from the right and left hepatic ducts and extending intrahepatically. Patients with intra-and extrahepatic metastasis were also deemed unresectable.

The benign biliary diseases group consisted of 92 patients and included 26 patients with benign bile duct stricture, 36 patients with cholecystolithiasis, 20 patients with cholecystic polyp, and 10 patients with chronic cholecystitis. The other control group consisted of 15 healthy individuals without any disease.

Methods

Blood samples obtained from patients were stored at -20 ℃ until used. CA19-9 and CEA were assayed by means of an immunoradiometric method with a commercially available CA19-9 RIA diagnostic kit (ELISA-CA19-9, CIS Bio International, France) and CEA RIA diagnostic kit (CIS Bio Industries, Gif-Sur-Yvette, France). Cutoff values recommended for diagnostic purpose were 37 KU· L^{-1} for CA19-9 and 22 μg· L^{-1} for CEA. Values above the cutoff concentrations were considered positive in this study. Sensitivity in detecting each group was compared between CA19-9 and CEA.

Statistics

The results were expressed as mean values ± standard deviation of the mean (mean±SD). Statistical analysis was performed using a statistical program (SPSS, 11.0 Inc, Chicago, IL). Statistical significance in mean values was evaluated by the Student' s *t* test. The one-way analysis of variance (ANOVA) was used to compare the different groups, and the Mann-Whitney rank sum test was used for intergroup comparisons. The relationships between CA19-9 and CEA, total bilirubin, alkaline phosphatase, or AST were determined by linear regression analysis.

RESULTS

Patients' characteristics

The Table 1 shows some of the characteristics of the different groups. Patients with cholangiocarcinoma were significantly older than either patients with benign biliary diseases or healthy individuals. However, the mean total serum bilirubin and serum alkaline phosphatase values were significantly higher in patients with cholangiocarcinoma, compared to the other two groups ($P<0.05$). Thus, the patients with cholangiocarcinoma had a more marked cholestatic profile than the other two groups of patients.

Serum CA19-9 and CEA values in cholangiocarcinoma, benign biliary diseases and healthy individuals

The mean CA19-9 and CEA concentrations were significantly greater in the cholangiocarcinoma group than those in the benign biliary diseases group or healthy individuals group (Figure 1). Table 1 shows the mean serum CA19-9 concentration in patients with cholangiocarcinoma was 290.31±5.34 KU· L^{-1} in comparison with 13.38±2.59 KU· L^{-1} in the benign biliary diseases group and 12.78±3.69 KU· L^{-1} in the healthy individuals group ($P<0.001$). Their corresponding mean serum CEA concentrations were 36.46±18.03 μg· L^{-1}, 13.84±3.85 μg· L^{-1} and 11.48±3.37 μg· L^{-1}, respectively ($P<0.05$).

Comparison of CA19-9 and CEA levels in serum in patients with cholangiocarcinoma and benign diseases

Table 2 shows the distribution of serum CA19-9 and CEA values in patients with cholangiocarcinoma and benign biliary diseases. Of the 35 patients with cholangiocarcinoma, 27(77.14%) had a concentration exceeding 37 KU· L^{-1} in serum CA19-9. Using a CA19-9 concentration of 37 KU· L^{-1}, the true negative rate of a CA19-9 for cholangiocarcinoma was 84.78% when

Table 1 Patients' characteristics in three groups (mean±SD)

Characteristics	Cholangiocarcinoma	Benign biliary diseases	Healthy individuals
N	35	92	15
Age (yr)	60.37±11.2	49.78±10.6	27.6±4.7
Sex (M / F)	19 / 16	39 / 53	15 / 0
Total bilirubin (umol / L)	47.4±2.1[a]	8.3±0.7	8.6±0.9
AST (U / L)	79±21.2[a]	26±3.5	21±4.6
ALP (U / L)	193±41.8[a]	114±50.6	90±18.3
CA19-9 (KU / L)	290.31±5.34[bc]	13.38±2.59	12.78±3.69
CEA (μg / L)	36.46±18.03[a]	13.84±3.85	11.48±3.37

[a]$P<0.05$, [b]$P<0.001$ *vs* benign biliary diseases or healthy individuals. [c]$P<0.05$ *vs* serum CEA. AST=aspartate aminotranferase, ALP=alkaline phosphatase, CA19-9=carbohydrate antigen 19-9, CEA=carcinoembryonic antigen.

Table 2 Comparison of serum CA19-9 and CEA levels in patients with cholangiocarcinoma and benign biliary diseases

	CA19-9[△]	CA19-9	CEA	CA19-9 or CEA	CA19-9 and CEA
Sensitivity(true positive)*	65.71%(23 / 35)	77.14%(27 / 35)[a]	68.57%(24 / 35)	91.43%(32 / 35)	62.86%(22 / 35)
Specificity(true negative)**	88.04%(81 / 92)	84.78%(78 / 92)	81.52%(75 / 92)	76.09%(70 / 92)	86.96%(80 / 92)
Positive predictive value	67.65%(23 / 34)	65.85%(27 / 41)	58.54%(24 / 41)	59.26%(32 / 54)	64.71%(22 / 34)
Negative predictive value	87.10%(81 / 93)	90.70%(78 / 86)	87.21%(75 / 86)	95.89%(70 / 73)	88.89%(80 / 90)
Accuracy	81.89%(104 / 127)	82.68%(105 / 127)	77.95%(99 / 127)	80.31%(102 / 127)	80.31%(102 / 127)
False positive rate	11.98%(11 / 92)	15.22%(14 / 92)	18.48%(17 / 92)	23.91%(22 / 92)	13.04%(12 / 92)

*Number of positive tests / number of patients with cholangiocarcinoma. **Number of negative tests / number of patients with benign biliary diseases. [a]$P<0.05$ *vs* CEA. [△]When the cutoff value of serum CA19-9 was 100 KU· L^{-1}.

assessed as the benign biliary diseases. The sensitivity (true positive) and specificity (true negative) of CEA were 68.57% and 81.52%, respectively (Table 2). Among the 92 patients with benign biliary diseases, the false positive rates of serum CA19-9>37 KU· L^{-1} and CEA>22 μg· L^{-1} were 15.22% (14/92) and 18.48% (17/92), respectively. The combination of CA19-9 and CEA showed the highest sensitivity and specificity, they were 91.43% and 76.09% when CA19-9 or CEA was positive, and they were 62.86% and 86.96% when both CA19-9 and CEA were positive, respectively. If the cutoff value was increased from 37 KU· L^{-1} to 100 KU· L^{-1}, the sensitivity and specificity of CA19-9 were 65.71% (23/35) and 88.04% (81/92), respectively.

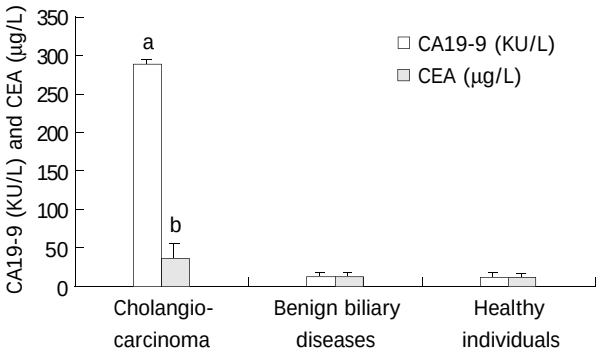

Figure 1 Mean serum CA19-9 and CEA concentrations. [a]*P*<0.001, [b]*P*<0.05 *vs* benign biliary diseases or healthy individuals. [c]*P*<0.05 *vs* serum CEA.

Correlation between serum CA19-9 and total bilirubin, ALP, AST or CEA in patients with cholangiocarcinoma

In patients with cholangiocarcinoma, no correlation was found between CEA and CA19-9 concentration (*r*=0.036). Likewise, no correlation was found among serum CA19-9, or ALP, AST, and total bilirubin (*r*=0.015, *r*=0.037 and *r*=0.145, respectively). Thus, elevated serum CA19-9 value could not be attributed to either cholestasis or hepatocellular injury.

Decrease of serum CA19-9 values in patients with cholangiocarcinoma after undergoing curative resection

Of the 35 patients with cholangiocarcinoma, 15 patients underwent curative resection and 20 had unresectable cancer. The mean preoperative and postoperative serum CA19-9 levels in the respectable group were 286.41±4.36 KU· L^{-1} and 62.01±17.43 KU· L^{-1}, respectively. The corresponding indexes of serum CEA were 39.41±24.35 μg· L^{-1} and 28.69±11.03 μg· L^{-1}, respectively (Figure 2 and Table 3). Thus, patients after curative resection had significantly lower mean serum CA19-9 and CEA concentrations than those before operation (*P*<0.001 and *P*<0.05). The mean serum CA19-9 concentration in the respectable group was 286.41±4.36 KU· L^{-1} as compared to 391.37±5.76 KU· L^{-1} in the unresectable group (Figure 2 and Table 3). Thus, patients with unresectable disease had a significantly higher mean serum CA19-9 concentration than those with respectable disease (*P*<0.05).

Table 3 Preoperative and postoperative change of serum CA19-9 and CEA concentrations in patients with cholangiocarcinoma undergoing curative resection (mean±SD)

Groups	CA19-9 (KU/L)	CEA(μg/L)
After the resectable (*n*=15)	62.01±17.43[a]	28.69±11.03[c]
Before the resectable (*n*=15)	286.41±4.36	39.41±24.35
The unresectable (*n*=20)	391.37±5.76[b]	43.28±20.68

[a]*P*<0.001, [b]*P*<0.01, [c]*P*<0.05 *vs* before the respectable group.

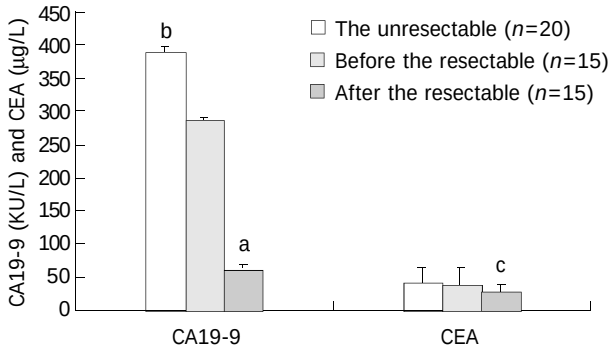

Figure 2 Mean postoperative serum CA19-9 and CEA levels of resectable group and mean serum CA19-9 and CEA levels of the unresectable group.

Relation between serum CA19-9 and CEA values and clinicopathologic features in 35 patients with cholangiocarcinoma

Perineural invasion by malignant cells was found in 23 of the 33 patients and more frequently encountered in cancer of the upper portion of extrahepatic bile ducts. Of the 35 tumors, lymph node metastasis was seen in 18 and venous invasion in 17. Histologically, 35 examples of cholangiocarcinoma were composed of 16 well-differentiated adenocarcinomas, 12 moderately-differentiated adenocarcinomas, 5 poorly-differentiated adenocarcinomas, 1 anaplastic carcinoma, and 1 adenosquamous carcinoma (Table 4). Table 4 shows statistically significant differences in serum CA19-9 and CEA between positive and negative lymph node metastases (*P*<0.01, *P*<0.05), perineural invasion (*P*<0.01, *P*<0.05), and venous invasion (*P*<0.05). However, there appears to be no relationship between the degrees of differentiation of carcinomas and the values of serum CEA and CA19-9.

Table 4 Relation between serum CA19-9 and CEA levels and clinicopathologic features of tumors in 35 patients with cholangiocarcinoma (mean±SD)

	No. of patients	CA19-9 (KU/L)	CEA (μg/L)
Well-differentiated adenocarcinoma	16	261.67±6.31	34.37±11.29
Moderately-differentiated adenocarcinoma	12	270.56±4.57	38.42±19.23
Poorly-differentiated adenocarcinoma	5	302.71±5.81	48.23±23.64
Anaplastic carcinoma	1	468	64
Adenosquamous carcinoma	1	293	27
Perineural infiltration			
Positive	23	310.12±7.11[a]	37.64±10.35[b]
Negative	10	195.16±6.48	21.28±13.15
Venous invasion			
Positive	17	318.49±3.27[b]	39.78±18.41[b]
Negative	18	253.08±5.37	29.67±20.13
Lymph node metastasis			
Positive	18	345.66±4.23[a]	46.37±9.46[b]
Negative	17	265.30±4.58	31.89±16.46

[a]*P*<0.01, [b]*P*<0.05 *vs* negative groups.

DISCUSSION

Since koprowski *et al*.[19] discovered CA19-9 from the human colon cancer cell line, which has been commonly used in the

diagnosis of pancreatic and biliary malignant diseases. Recently, its usefulness has been demonstrated in the staging, evaluation of resectability, and assessment of prognosis and recurrence, as well as in the initial diagnosis of malignant disorders, especially pancreatic cancer[31-37].

Elevated concentration of serum CA19-9 (>37 KU· L^{-1}) in bile duct cancers has been frequently reported. Torzilli *et al.*[38] described a positive rate of 97% in patients with cholangiocarcinoma, whereas Hultcrantz *et al.*[39] described a rate of 76%, and Caturelli *et al.*[40] described a 68% rate. We have also found an elevation of CA19-9 in 77.14% of cholangiocarcinomas, which was comparable to the rates reported in other studies. Furthermore, as shown in Table 2, the application of CA19-9 for differentiating cancer from benign biliary diseases was inspiring in our study because of its high sensitivity and specificity. The sensitivity and specificity in our study were 77.14% and 84.78%, respectively, which were similar to 78.2% and 81.4% reported by kau *et al.*[41]. However, the concentration of CA19-9 could raise in patients with benign inflammatory conditions as well as in malignant disease[42-45]. Indeed, Ahrendt *et al.*[46] reported a moderate increase in CA19-9 concentration in 13.8% of patients with benign biliary tract diseases. The positive serum rate was 28% in patients with acute cholangitis, as reported by Ker *et al.*[47], and 35.6% in patients with bile duct stones, as reported by Shiozawa *et al.*[48]. In our present study, an increased concentration of CA19-9 was found in 15.22% of patients with benign biliary diseases. According to Ker *et al.*[47], CA19-9 was synthesized by normal biliary ductal cells and malignant cells. If bile flow is blocked by biliary obstruction in benign conditions, such as choledocholithiasis, epithelial cells will be markedly impaired by inflammation and will proliferate concurrently. As a result, more CA19-9 may be secreted and leaked out into the bloodstream. An elevated CA19-9 level often returns to normal with appropriate decompression of the common duct or relief of acute cholangitis. However, the increment of CA19-9 in benign diseases is usually not significant, and a concentration of CA19-9 exceeding 480 KU· L^{-1} is rare. In our study, only 2(2.17%) patients with benign biliary diseases exhibited CA19-9 levels >480 KU· L^{-1}.

CEA is mainly secreted by digestive glandular cancers and their metastases. It has also been found in other types of cancer such as breast, lung, ovary, thyroid cancers. The sensitivity and specificity of serum CEA in our study were 68.57% and 81.52%, respectively, which were similar to 63.3% and 78.4% reported by Ramage *et al.*[49]. The false positive rate of serum CEA was 18.48% (14/92). Serum CA19-9 was obviously superior to serum CEA in the diagnosis of cholangiocarcinoma and often considered the standard marker for pancreatic cancer and cholangiocarcinoma, with which other markers were compared[50-53]. Our data also showed a much higher sensitivity with serum CA19-9 than with serum CEA in detecting cholangiocarcinomas, but the sensitivity and specificity could be raised by combining these two tumor markers. Furthermore, no correlation between the levels of serum CEA and CA19-9 was found (r=0.036).

Serum CA19-9 has been reported to be able to predict resectability of cholangiocarcinoma[54-57]. In our study, marked elevation of serum CA19-9 tended to associate with advanced and unresectable biliary cancers. Both serum CA19-9 and CEA levels had a positive correlation with tumor stage. This positive correlation is theoretically helpful in assessing the therapeutic effect and monitoring tumor recurrence after treatment. CA19-9 and CEA have been claimed to have a prognostic value in cholangiocarcinomas[7,21,24,30,58]. When applied to cholangiocarcinomas, both tumor markers could provide valuable prognostic information. In our study, serum CA19-9 and CEA concentrations were measured in 15 of 35 patients with cholangiocarcinoma after undergoing curative resection. Within two weeks after operation, the average concentration of serum CA19-9 and CEA was 62.01±17.43KU· L^{-1} and 28.69±11.03 μg· L^{-1}, respectively, with an obvious decrease compared with those before operation (P<0.001 and P<0.05). Hence, serum CA19-9 could provide more important diagnostic and prognostic values than CEA in cholangiocarcinoma.

In summary, serum CA19-9 is an effective tumor marker in diagnosing cholangiocarcinoma, deciding whether the tumor has been radically resected and monitoring the effect of treatment. Serum CA19-9 determination is a useful adjunct in our diagnostic armamentarium for cholangiocarcinoma. However, serum CA19-9 has limitations in diagnosing cholangiocarcinoma. Indeed, our data clearly demonstrated that a negative test could not exclude cholangiocarcinoma. Moreover, for a patient suspected of having biliary cancer, consideration of the presence of acute cholangitis or cholestasis is suggested. If the patient has no evidence of acute cholangitis or cholestasis, a cutoff value of 37 KU· L^{-1} may be appropriate. However, if a patient shows symptoms and signs of acute cholangitis or cholestasis, application of CA19-9 should be delayed until after recovery from acute conditions or a cutoff value of 100 KU· L^{-1} should be used. Additional bile-or serum-based tests are needed in the diagnosis of cholangiocarcinoma. Ultimately, a profile of tests analogous to liver biochemistry measurement of serum or bile tumor markers and genetic analysis for cholangiocarcinoma-associated mutations will need to be developed to assist in the diagnosis of this disease.

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• BRIEF REPORTS •

Extraction of protoporphyrin disodium and its inhibitory effects on HBV-DNA

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Abstract

AIM: To explore an ideal method for extracting protoporphyrin disodium (PPN) from unanticoagulated animal blood, and to study the inhibitory effects of PPN on HBV-DNA duplication and its cytotoxicity to 2.2.15 cell strain.

METHODS: Protoporphyrin methyl ester and other intermediate products were prepared with protoheme separated from protein hydrolysates of coagulated animal blood, which were finally made into PPN and detected quantitatively with an ultraviolet fluorescent analyzer. Ten μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml of PPN-aqueous solution were added into culture medium for 2.2.15 cells respectively. Eight days later, the drug concentration in supernatant from the culture medium was detected when inhibition rate of HBeAg, cell survival rate when inhibition rate of HBeAg was 50% (ID50), and when survival cells in experimental group were 50% of those in control group (CD50), and the therapeutic index (TI) was also detected. PPN with different concentration of 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml was respectively mixed and cultivated with HepG2 2.2.15 cell suspension, and then the inhibition of PPN against HBV-DNA was judged by PCR.

RESULTS: The extract of henna crystal was identified to be PPN. When the concentrations of PPN were 160 μg/ml and 80 μg/ml, the inhibition rates of HBeAg were 89.8% and 82.4%, and the cell survival rates were 98.7% and 99.2%.

CONCLUSION: It is suggested that PPN can be extracted from unanticoagulated animal blood. PPN can inhibit HBV-DNA expression and duplication *in vitro*, and has no cytotoxicity to liver cells. Further study and application of PPN are warranted.

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INTRODUCTION

Protoporphyrin disodium (PPN) is a macrocyclic compound with a conjugated double bond, which is consisted of four pyrrole rings connected by four methylene bonds, and is a derivative of porphine. PPN can be prepared with extracted haemachrome originated from ferrohemoglobin in serum and other chemicals. PPN is a pharmaceutics to improve liver functions and can be used in clinical therapy for recovery of injured liver cells.

PPN could be obtained from fresh anticoagulant animal blood according to reports[1-3]. But the PPN in this study was obtained from fresh unanticoagualted animal blood, and the method has not been reported in the literature.

Hepatitis B is widely occurred in China, and severely affects people' s health and the quality of life. In order to find out the relationship between PPN and the expression and duplication of HBV-DNA, hoping to seek for an effective therapy of Hepatitis B, we designed an experimental *in vitro* study.

MATERIALS AND METHODS

Reagents

Unanticoagulated pig blood (from Huainan area), NaOH, zinc powder, chloroform, skellysolve G, 2.2.15 cell strain (from the Institute of Infectious Disease, Peking Medical University), kit for ELISA (from Huamei Biological Engineering Co.,Ltd), kit for PCR (from Huamei Biological Engineering Co.,Ltd).

Instruments

Tissue homogenate instrument (from Shanghai Biaomo), magnetic stirring apparatus, glassy device of reflux, glassy filter, chromatography column with neutral aluminium oxide (from Shanghai-Jinhua Chromatography Equipment Factory), drying oven by electrothermal blow (form Shanghai-Yuejin Equipment Factory), gamma radio immunoassay counters (from State-Operated Factory 262), CO_2 incubator (from Japan), SLT-Spectra-I enzyme analyzer (from America), DNA amplifier (from Zhuhai Hema Bio-Tech Institute), ultraviolet fluorescent analyzer (from Shanghai-Kanghua Biochemical Instrument Manufactory).

Methods

Extraction of protoheme 1L homogenate of unanticogulated pig bloods was added to a container, and 100 g NaOH was added simultaneously during stirring until they were well mixed. When the mixture changed into dilute solution after placement for 24 h, it was heated and stirred by magnetic stirring apparatus at the 80 ℃ to 90 ℃ for 12 hours. When the mixture was naturally cooled to 60 ℃, 1M HCL aqueous solution was added into the mixture till precipitation of protoheme occurred. The precipitation was taken out, washed 3 times with water and dried. Finally about 1.7g of protoheme was obtained.

Preparation of crude protoporphyrin 1.5 g zinc powder divided into six portions was added into the mixture of 250 ml formic acid aqueous solution (85%) and 5 g protoheme under the condition of heating, stirring and regurgitating respectively. Each addition had an interval of 5 minutes. Then they were heated and regurgitated once more for 20 min and then cooled and filtered. Ammonium acetate aqueous solution (20%) was

added into the filtrate, then the solution was filtered when crystals occurred after placement for 12 h. After the crystals were dissolved with 600 ml ammonia water (2%), ammonium acetate aqueous solution (30%) was added into the solute and placed for 12 h till crystallization occurred. The crystals were filtered out and washed 3 times with 600 ml ammonium acetate aqueous solution (2%) and 90 ml distilled water, respectively. Then it was dried into crude protoporphyrin, which was a kind of brown cystalline materials of about 2 g.

Preparation of protoporphyrin methyl ester 5 g crude protoporphyrin was dissolved in 200 ml hydrochloric methanol aqueous solution (1%), and the reactants were heated, regurgitated for 20 min. Then 1 000 ml ammonium carbonate aqueous solution (1%) was added into the above cooled reactants and placed till crystallization occurred. The crystalline materials were taken, and washed 3 times with distilled water, then dissolved in 200 ml chloroform and the solute was taken to pass through chromatography column with 1 000 g neutral aluminium oxide. After the elution with prepared eluting agent of chloroform and petroleum ether with volumetric proportion at the ratio of 1 to 3 was completed, the eluent of protoporphyrin methyl ester was collected and evaporated, and a kind of brown cystalline materials was obtained. Then the brown cystalline materials were crystallized 2 times with chloroform and methanol, and 2 g protoporphyrin methyl ester was acquired.

Preparation of pure protoporphyrin 5 g protoporphyrin methyl ester was dissolved in 150 ml HCL aqueous solution (25%). After placed for 8 h, the reactants were neutralized with NaOH aqueous solution (30%), then crystallization occurred. The crystalline materials were isolated and washed 3 times with distilled water. After being dried, the crystalline materials were re-crystallized 2 times with pyridine, and 2.5 g brown pure protoporphyrin was obtained.

Preparation of PPN The mixture of 10 g pure protoporphyrin, 5 g NaOH and 500 ml anhydrous alcohol was heated and regurgitated for 2 h. Crystalline materials occurred after the mixture was cooled. Then the crystalline materials were isolated, washed 3 times with anhydrous alcohol and dried. Finally, 9 g brown crystalline materials of PPN was obtained.

Inhibitory effects of PPN on HBV-DNA expression *in vitro* 2.2.15 cell strain was prepared based on human cancer cell strain of the liver, $HepG_2$, which had been infected with HBV-DNA, could express all marks of duplications of HBV-DNA effectively[4-17]. Regarding 2.2.15 cell strain as the target cell, the effect of PPN on HBV-DNA was confirmed by detecting the levels of HBeAg in culture supernatants with quantitative methods.

Procedure was as follows: 1×10^5/ml cell suspension was prepared with 2.2.15 cell strain which grew well. One ml cell suspension was added into each of the 24-hole plastic plate respectively and cultivated. After incubation at 37 ℃ for 48 h, fresh culture media with different concentrations of PPN (10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, 160 μg/ml) were then replaced every 3 days. Eight days later, supernatants were collected and stored at 20 ℃ for detection of HBeAg levels and cytotoxicity of PPN to liver cells. ELISA was used for detection of HBeAg. The detailed procedure followed the operating instructions. Inhibition rate (%)=(P/N values of the control holes- P/N values of the study holes)/(P/N values of the control holes -2.1)×100%, and ID_{50} represented the concentration of PPN when the inhibition rate of HBeAg was 50%. The cytotoxicity of PPN to liver cells was detected by MTT to determine the survival rate of liver cells. The survival rate of liver cells (%)=values of the study holes (A_{595}-A_{650})/ values of the control holes (A_{595}-A_{650})×100%. CD_{50} represented the concentration of PPN when the number of the survival cells in the detected holes to the survival cells in the study holes was 50%.

Inhibitory effects of PPN on HBV were evaluated with TI ($TI=CD_{50}/ID_{50}$). When TI<1, PPN was cytotoxic and poorly effective on liver cells. When 1<TI<2, PPN was cytotoxic and effective. When TI≥2, PPN was mildly cytotoxic and effective. The higher the values of TI, the greater the inhibitory effects of PPN on HBV and the less the cytotoxicity of PPN to liver cells[18,19] (Table 1).

Inhibitory effects of PPN on HBV-DNA duplication *in vitro* PPN was diluted to a series of solutions with different concentrations (including 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml) by aseptic techniques for further use. $HepG_2$ 2.2.15 cells in good cultivation were dispensed to the suspension with a concentration of 1×10^5/ml, 6 identical shares of it were subsequently taken out, PPN with the concentration of 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml respectively was added to 5 of 6, the remaining one had no PPN. After a period of cultivation, the cells were collected, then whole genome DNA of them was extracted by using CASSupper blood genomic DNA isolation kit and further amplified by PCR. The amplification system included 10×buffer 25 ul, dNTP 3 ul, sense 0.5 ul, anti-sense 0.5 ul, TaqE 1.5 ul and template 2 ul. The parameters included at 95 ℃ for 5 min, at 94 ℃ for 30 s, at 55 ℃ for 30 s, at 72 ℃ for 30 s, 35 cycles and extension at 72 ℃ for 5 min. The final products with DGL-2000 were further performed with routine electrophoresis. During the process of analysing by using agarose gel electrophoresis containing EB, each hole was added 10 ul of products and placed at 80-100V for 20 min. Lastly, the agar plates were observed by ultraviolet fluorescence analysis.

RESULTS

General characterisics of PPN

The prepared PPN was brown crystalline materials, soluble in water, slightly soluble in methyl alcohol, but not soluble in chloroform, diethyl ether or dimethy ketone. PPN displayed absorption peaks at wavelengths of 600 nm, 556 nm and 408 nm, and the maximal absorption wavelength was 408 nm. When exposed to ultraviolet, strong fluorescence could be observed in red[3].

Quantitative detection of PPN

According to the fact that when PPN was dissolved in 1.37M HCL and placed at wavelength of 408 nm, the $E_{1cm}^{1\%}$ was 4.81×10^3, the purity of PPN extract was detected to be 97.3%.

Inhibitory effects of PPN on HBV-DNA in vitro

Table 1 Inhibition of PPN on HBV-DNA expression *in vitro*

PPN (μg / ml)	OD	Inhibition rate of HBeAg (%)	Survival rate of liver cell (%)
160	1.20	89.8	98.7
80	1.30	82.4	99.2
40	1.50	56.7	100
20	1.85	32.3	100
10	2.30	5.5	100

CD_{50}=279.4 μg / ml, ID_{50}=37.4 μg / ml, TI=7.47.

Table 1 shows that when the concentrations of PPN were 160 μg/ml and 80 μg/ml, the inhibition rates of HBeAg were 89.8% and 82.4% respectively and cellar survival rates were 98.7% and 99.2%, TI=7.47.

It was obvious that the luminance of amplified bands of samples without addition of PPN was the brightest, while that of samples with addition of PPN decreased gradually accompanying increased concentration of PPN.

DISCUSSION

While fresh anticoagulated animal blood was used as raw materials to prepare PPN, the harvest, transportation, and preservation of the raw materials needed a strict condition, which made the source of raw materials limited, working cost increased and the scale of production limited. In this study, unanticoagulated animal blood was used as raw materials to prepare PPN, that is, the form of the raw materials was improved. Using unanticoagulated animal blood as raw materials to prepare PPN, fine PPN could be obtained. The method is an ideal way of preparing PPN at present. Meanwhile, it can solve the problems mentioned. In addition, during the whole process of preparation, the special, expensive reagents are not necessary and also some reagents can be retrieved for reuse. The method is simple and convenient in its process of preparation and its production cost is lower. If some of amino acids produced during the preparation were studied, analyzed and purified, the additional value of the raw materials would be increased, so that large amount of animal blood resource can be fully utilized.

Protoporphyrin is the necessary component of ferrohemoglobin, myohemoglobin, cytochrome, catalase and tryptophan pyrrolase in human body. Protoporphyrin showed a circular structure in somatic cells, and could easily combine with metallic ion to form metallic porphyrin[21]. In the case of liver disease, liver functions might be in disorder, porphyrin and metallic porphyrin in bile decrease as well as the activity of catalase in liver would decrease. Therefore, PPN could activate the biosynthesis of porphyrin and metallic porphyrin in cells as demonstrated by *in vitro* test, increase the quantity of metallic porphyrin in cells, inhibit the decrease of activity of catalase, improve cellular respiration and regeneration, and decrease the necrosis of liver cells with recovery of its function. Based upon the above metabolic properties, PPN could improve blood flow and utilization rate of oxygen by liver and other related organs, accelerate the respiration of histocytes, improve metabolism of proteins and carbohydrates, enhance effects of complement fixation, and enhance immunity, anti-inflammation and anti-anaphylaxis potentials of the organism. Thus, PPN has been constantly studied and applied as an improving agent of liver functions[22-32]. At present, PPN is being studied for its antineoplastic effects in foreign countries. Inhibitory effects of PPN on expression and duplication of HBV-DNA *in vitro* was studied by us from another point of view. As table 1 shows, PPN could inhibit expression of HBV-DNA *in vitro* and had no cytotoxic action, and it is not difficult to see that the luminance of amplified bands decreased gradually with increased concentration of PPN. The mechanism of action in the above results might be that some intermediary metabolites of PPN in liver cells could affect antisense oligonucleotides (ASONs) located at pre-C and C gene regions of HBV and also adjust genes ENH I of HBV through ASONs. Then it could finally inhibit the duplication of HBV-DNA and the expression of HBeAg in host cells[33-35]. Therefore, further study and application of PPN in clinical therapy of hepatitis B should be carried out.

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• BRIEF REPORTS •

Mechanism of intrauterine infection of hepatitis B virus

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Abstract

AIM: To explore the possible mechanism of intrauterine infection of hepatitis B virus (HBV).

METHODS: HBV DNA was detected in vaginal secretion and amniotic fluid from 59 HBsAg-positive mothers and in venous blood of their newborns by PCR. HBsAg and HBcAg in placenta were determined by ABC immunohistochemistry.

RESULTS: The rate of HBV intrauterine infection was 40.1% (24/59). HBV DNA was detected in 47.5% of amniotic fluid samples and 52.5% of vaginal secretion samples respectively. HBsAg and HBcAg were detected in placentas from HBsAg-positive mothers. The concentration of the two antigens decreased from the mother's side to the fetus's side, in the following order: maternal decidual cells > trophoblastic cells > villous mesenchymal cells > villous capillary endothelial cells. However, in 4 placentas the distribution was in the reverse order. HBsAg and HBcAg were detected in amniotic epithelial cells from 32 mothers.

CONCLUSION: The main route of HBV transmission from mother to fetus is transplacental, from the mother side of placenta to the fetus side. However, HBV intrauterine infection may take place through other routes.

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INTRODUCTION

Hepatitis B virus infection is a worldwide health problem. China is one of the high prevalent areas, with a positive rate of HBsAg in population more than 10%[1]. Recent data indicate that the rate of intrauterine infection of HBV is 10%-44.4%[2,3]. Intrauterine infection is one of important routes of HBV transmission, and the main cause of HBV chronic infection. To explore the possible mechanism of intrauterine infection, we detected HBV DNA in the vaginal secretion and amniotic fluid from HBsAg-positive mothers and venous blood from the neonates by PCR, and also detected the distribution of HBsAg and HBcAg in the placenta by ABC immunohistochemical method.

MATERIALS AND METHODS

Patients

Pregnant women who gave birth in the Hospital of Women' s and Children' s Health Care in Zhaoqing, Guangdong Province, China and their full-term newborns were recruited into this study. All the mothers received a regular prenatal examination in the clinic during pregnancy, and were detected for HBV serum markers (HBVM) by ELISA. Fifty-nine HBsAg-positive mothers and their newborns were studied, 10 HBsAg-negative mothers and their newborns served as control. All the mothers had no threatened abortion or related history, no pregnancy related complications. There was no difference in age, pregnant frequency of mother and gestational age of fetus between the two groups. Sixty-nine mothers gave birth to sixty-nine newborns.

HBV DNA detection

Vaginal secretion of mothers was taken before amnion rupture. After entering labor of the mother, at a proper time or just after amnion rupture, amniotic fluid was taken, and strictly prevented from blood contamination. After birth, 3 ml of neonatal venous blood was taken and separated for serum. All specimens were stored at -20 ℃ and HBV DNAs were detected simultaneously. PCR test kits were purchased from Hua Mei Biological Engineering Company. The tests were performed strictly according to the manufacturer' s instructions.

Determination of HBsAg and HBcAg in placenta

Placental tissues of 1 cm×1 cm×2 cm, were taken from the fetal side and the maternal side respectively, fixed in 10% formalin, embedded with paraffin according to routine procedure and sliced in 5 μm thickness. Rabbit McAb against HBcAg and mouse McAb against HBsAg were used for immunohistochemical test and DAB staining kits were purchased from Wuhan BoShide Biological Engineering Ltd Company. HBsAg and HBcAg positive livers from autopsy were used as positive control, and placentas from HBVM negative mothers served as negative control. At the same time, we used PBS instead of the first antibody as blank control. Dark brown yellow in cytoplasm or nucleus was regarded as strongly positive, brown yellow as positive, and light brown yellow as weakly positive.

Diagnosis of intrauterine infection

The presence of HBV DNA in neonatal venous blood was regarded as intrauterine infection.

RESULTS

HBV DNA status in amniotic fluid, vaginal secretion and neonatal venous blood

The positive rates of HBV DNA in amniotic fluid and vaginal secretions were 47.5% (28/59) and 55.9% (31/59) respectively. No HBV DNA was detected in amniotic fluid and vaginal secretion in control group. Of the 59 newborns born to mothers with HBsAg-positive, 24 were HBV DNA positive in neonatal venous blood, the rate of intrauterine infection was 40.1%

(24/59). No HBV DNA was detected in neonatal venous blood from newborns whose mothers were HBsAg-negative.

HBsAg and HBcAg in placenta

The positive rates of HBsAg and HBcAg in placentas from 59 mothers with HBsAg- positive were 81.4% (48/59) and 61.1% (36/59) respectively. HBsAg in placenta appeared as inhomogeneous dark brown yellow granules in plasma of all kinds of cells. It was 76.27% (45/59) in maternal decidual cells, 72.88% (43/59) in trophoblastic cells, 62.71% (37/59) in villous mesenchymal cells, 52.54% (31/59) in villous capillary endothelial cells, 54.24% (32/59) in amniotic epidermic cells. The positive coloration of HBsAg was seen in part of the villous interstices. The distribution of positive cells was patchy or conglomerate. The stained HBcAg in placentas was homogeneous granule, existing in nuclei of the positive cells which were distributed in focus or dispersion, mainly including 59.32% (35/59) of maternal decidual cells, 55.93% (33/59) of trophoblastic cells, 50.85% (30/59) of villous mesenchymal cells, 44.07% (26/59) of villous capillary endothelial cells, 49.15% (29/59) of amniotic epidermic cells. No HBsAg or HBcAg was detected in placentas of the control. The number of positive cells of HBsAg or HBcAg and the degree of staining were gradually decreased from maternal decidual cells to villous capillary endothelial cells.

On the contrary, the distribution of HBsAg- and HBcAg-stained cells in 4 placentas was gradually decreased from villous capillary endothelial cells to maternal decidual cells. The staining degree of HBsAg and HBcAg was also decreased in the same order.

DISCUSSION

Most researchers hold that the mechanism of HBV intrauterine infection is transplacental infection. In 1987, Lin detected 32 placentas of HBsAg and HBcAg positive mothers using PAP immunohistochemistry, and did not find HBsAg[4]. Tang detected HBV DNA in placentas of induced labor from HBsAg-positive mothers using dot blot hybridization, and found HBV DNA in 2 cases[5]. Lucifora detected 12 placentas of HBsAg carriers with no symptoms by immunohistochemistry and found HBsAg and HBcAg in villous capillary endothelial cells[6,7]. Xu[8] and Yan[9] detected placentas from HBsAg-positive mothers by ABC immunohistochemistry and *in situ* hybridization and found HBsAg, HBcAg and HBV DNA in all kinds of placental cells. Wang *et al* detected 24 placentas of HBsAg and HBcAg positive mothers using *in situ* hybridization and found HBV DNA was mainly distributed in maternal decidual cells, while no HBV DNA- positive cells were in the villi[10]. The results above were different obviously. In the present study, by using PCR for the determination of intrauterine HBV infection and ABC immunohistochemistry for the detection of the presence of HBsAg and HBcAg in placenta, we detected 59 placentas of HBsAg-positive mothers and found the positive rates of HBsAg and HBcAg were 81.4% (48/59) and 61.1% (36/59) respectively. The detection rate of HBsAg and HBcAg, the proportion of positive cells and the degree of staining were gradually decreased from the maternal side to the fetus side of placenta (decidual cells > trophoblastic cells > villous mesenchymal cells > villous capillary endothelial cells). The villous capillary endothelial cells were infected by HBV in 31 mothers, from whom 22 newborns had HBV intrauterine infection. These results indicated that HBV could infect all kinds of cells in placenta, which was the possible mechanism of intrauterine infection that HBV infected cells from maternal decidua to villous capillary endothelia or that HBV infected trophoblastic cells directly, then to villous mesenchymal cells and villous capillary endothelial cells resulting in fetus infection.

In our study, the number of HBsAg- and HBcAg- positive cells was gradually decreased from villous capillary endothelial cells to maternal decidual cells in 4 placentas. The degree of staining was decreased in the same order from the fetus side to the mother side of placenta. HBV DNA was positive in 2 of the venous blood samples. This indicated that HBV infected the fetus first, and then infected cells in different layers of placenta. In these 4 cases HBsAg and HBcAg were detected in amniotic epidermic cells, HBV DNA in amniotic fluid and vaginal secretion was also detected, suggesting that the ascending infection from vagina might exist, that is to say, HBV in vaginal secretion infected fetal membrane, amniotic fluid, fetus and cells of different layers in placenta or HBV infected fetal membrane first then infected cells in different layers of placenta from the fetus side to the mother' s side.

From the 1980' s, researchers all over the world have proved that HBV DNA was existent in all generations of spermatogenic cells and sperms in HBV-infected males. Researchers studied male HBV carriers whose wives were not infected with HBV, and their fetuses. The results of HBV DNA sequencing showed that the homology between the father and his son or daughter was 98%-100%. Some researchers found HBsAg in follicular fluid of HBsAg-positive women by immunohistochemistry. Still others found HBV DNA in ovary from a woman who died of severe hepatitis using *in situ* hybridization. HBV DNA was mainly in plasma of ovum and interstitial cells. Now that human oocytes can be infected by HBV, the possibility of HBV transmission through oocytes may exist. In our study, although the fetus HBV infection through oocyte has not been proved in the 2 cases, we could not exclude the possibility.

In conclusion, intrauterine HBV infection is mainly transmitted through the placenta from the maternal blood to the fetus. HBV infection through vagina or oocytes may exist.

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Quantitative study of multiple biomarkers of colorectal tumor with diagnostic discrimination model

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Abstract

AIM: To evaluate the multiple biomarkers of colorectal tumor and their potential usage in early diagnosis of colorectal cancers.

METHODS: Multiple biomarkers (DNA contents, AgNOR, PCNA, p53, c-erbB-2) in 10 normal colorectal mucosae, 37 colorectal adenomas and 55 colorectal cancers were analyzed quantitatively in the computed processing imaging system. Discrimination patterns were employed to evaluate the significance of single and multiple indices in diagnosis of colorectal cancers.

RESULTS: The mean values of the analyzed parameters increased in order of the normal mucosa, adenoma and adenocarcinoma, and this tendency reflected the progression of colorectal malignancy. The parameters including DNA index, positive rates, densities of AgNOR, c-erbB-2, and p53, shape and density of nucleus were relatively valuable for diagnoses. Then a diagnostic discrimination model was established. The samples were confirmed with the model, the sensitivity rates in cancer group and adenoma group were 96.36% and 89.19%, respectively. The value of proliferating cell nuclear antigen (PCNA) in early diagnosis of colorectal cancers was uncertain.

CONCLUSION: The quantitative evaluation of some parameters for colorectal tumor can provide reproducible data for differential diagnosis. The established diagnostic discrimination model may be of clinicopathological value, and can make the early diagnosis of colorectal cancer possible.

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INTRODUCTION

Colorectal carcinoma is one of the most common malignant tumors worldwide. Quantitative changes in nucleus, DNA content, PCNA, AgNOR were observed in the progression of tumor[1-6]. Genes such as p53, *cerbB-2* might play a significant role in carcinogenesis[7-13]. Although the biologic parameters of tumors have been assessed extensively, measurement of these parameters has little impact on histological diagnosis. Furthermore, analysis of a single parameter is insufficient to evaluate tumor malignancy. Meanwhile, distinguishing benign from malignant lesions has traditionally been subjective; a quantifiable test is useful for the diagnosis of colorectal cancer. In this study, we used quantitative analysis to examine the potential usage of multiple biomarkers (DNA contents, AgNOR, PCNA, p53, c-erbB-2) in early diagnosis of colorectal cancer.

MATERIALS AND METHODS

Materials

Pathological specimens were obtained from Department of Pathology at Fujian Medical University from 1991 to 1996, including 55 cases of colorectal carcinoma, 37 cases of colorectal adenoma and 10 cases of normal colonic mucosae. The diagnosis was confirmed pathologically. The patients included 35 males, and 20 females with a mean age of 57±12.83 years (range 31 to 84 years). Histologically, there were 15 highly differentiated types, 19 moderately differentiated types, 21 poorly differentiated types, and 13 cases had a local lymph node metastasis and 1 case had the liver metastasis.

Reagents and methods

Schiff reagent, AgNOR staining fluid, antibodies against PCNA, c-erbB-2 and p53 and SP immunohistochemical reagent were purchased from Fujian Maxin Co.Ltd.

Formalin-fixed, paraffin-embedded specimens were cut in to 5 μm in thickness. The slices were stained with Feulgen, AgNOR and the detected antigen, and those in the control group were stained without primary antibody.

Semi-quantitative evaluation

Some silver stained black particles were observed in nuclei and classified. The positive staining rate was calculated[14]. A semi-quantitative evaluation system was used to determine the antigen expression in specimens[15]. Expression of p53 and c-erbB-2 was graded as the following scale: <10% "-",10-25% "+", 26-50% "++", 51-75% "+++", >75 "++++". Expression of PCNA was graded as the following scale: <25% "+", 26-50% "++", 51-75% "+++", >75% "++++".

Quantitative evaluation

The specimens were examined for multiple biomarkers with quantitative analysis using the computer processing image system (American Image Co. 8000 Type). Under the same power field of microscope, 150-200 cells were examined. Multiple biomarkers stained with silver or PCNA included DNA content, DNA index, nucleus area, Abs, granular shape factor, width and length of nuclei, the widest and longest diameter, and density of nucleus.

Statistical analysis

Student' s *t* test was used for analysis of variance. Discriminant analysis was done for multiple parameters and indices. The selected significant parameters were used to set up a diagnostic discrimination model. The SAS system for windows (version 6.12) was used for completing all the statistical analyses.

RESULTS

Multiple biomarkers (DNA contents, AgNOR, PCNA, p53, c-erbB-2) were quantitatively processed using the computer processing image system in 10 normal colonic mucosae, 37 colorectal adenomas and 55 colorectal carcinomas. The values of most parameters were increased in the order of normal mucosa, adenoma and adenocarcinoma. The tendency reflected the progression of colorectal malignancy. But the PCNA content was peaked in adenoma and decreased in carcinoma. The values were subjected to discrimination pattern method to evaluate the significance of parameters in multiple indices in order to set up the discrimination model. Then the model was used to recheck the samples. DNA index, shape factor, the widest diameter and density of nuclei were demonstrated to be the valuable parameters in feulgen-stained sections. The concordance rate was 86.02% for cancer group and 80.06% for adenoma group. The relatively valuable parameters in silver stained slides were average number, positive rate, density and aspect factor of particles. The concordance rate of the established model was 79.57% for cancer group and 78.65% for adenoma group. The valuable parameters in PCNA stained specimens were positive rate and density, and the concordance rate was 76.34% for cancer group and 73.55% for adenoma group. The valuable parameters in c-erbB-2 stained samples were Abs, positive rate and density, the concordance rate was 90.32% for cancer group and 90.14% for adenoma group. The valuable parameters in p53 stained positive samples were total positive area and density, and the concordance rate was 86.02% for cancer group and 83.55% for adenoma group. Discriminant analysis was performed for the above parameters. DNA index (X_1), positive rate (X_2), density (X_3) and aspect factor of AgNOR (X_4), density of c-erbB-2 (X_5), density of p53 (X_6), shape factor (X_7) and density of nucleus (X_8) were demonstrated to be the relatively valuable indices. Then a diagnostic discrimination model was established, and the model rechecked the samples, the concordance rates in cancer group and adenoma group were 96.36% and 89.19%, respectively. The models were as follows.

$Y(1)=-412.86-7.30X_1+34.83X_2+1.15X_3+2.54X_4+500.07X_5+17.34X_6+2.54X_7+409.68X_8$

$Y(2)=-480.40-7.04X_1+34.33X_2+0.98X_3+2.81X_4+560.55X_5+26.85X_6+2.47X_7+375.14X_8$

$Y(3)=-432.76-4.58X_1+41.97X_2+0.75X_3+2.63X_4+529.44X_5+52.13X_6+2.26X_7+321.11X_8$

DISCUSSION

Relationship among DNA content, shape parameters of nucleus and colorectal carcinoma

Most tumor cells had a certain amount of abnormal DNA; DNA content of tumor was closely related to biological behaviors[1,2]. Our study showed that the level of DNA content was increased in the order of normal mucosa, adenoma and adenocarcinoma with different significances. It has been confirmed that DNA content is a reliable and objective marker in early diagnosis of colorectal carcinoma and in distinguishing benign from malignant tumors.

In diagnosis of colorectal carcinoma with quantitative analysis as reported[1,2], the results varied with selected parameters. We believed that abnormalities of nuclei were the most important phenomena in hypertrophy of neoplastic cells in addition to abnormal structure of cells or tissues. It is well known that increased mitotic nuclei, multi-hierarchical structure, enlargement and pleomorphism of nuclei are expressed in most rapidly growing neoplasms. We suspected that increased nucleus area and much more irregular shape of nucleus were in accordance with the normal mucosa-adenoma-adenocarcinoma sequence. Parameters of nuclear shape and density of DNA reflected the malignancy of tumor significantly.

Although we considered that area of nucleus would play an important role in diagnosis of tumor with three-dimensional (3D) image processing, this study failed to show that areas of nuclei could reflect hyperplasic degree of tumor quantitatively. This might be due to the limitation of bi-dimension that could not reveal the whole nucleus.

Previous studies reported that Abs was an objective parameter to reflect the nucleus. But discriminant analysis showed that Abs was not relatively valuable. While Abs and density of nucleus had significant differences among the groups. We reason that sectional shape of nucleus is different from its real shape, so average area of Abs (density of nucleus) reflects of DNA content more objectively.

Relationship between AgNOR and colorectal carcinoma

Variation of the number of nucleolar organizer regions (NORs) could reveal the conditions of cellular activity[16-20]. It was suggested that AgNOR dot count of cells had a potential role in distinguishing benign from malignant tumors and in their early diagnosis[21]. Our results revealed that the count of AgNOR was increased in accordance with the normal mucosa-adenoma-adenocarcinoma sequence. Due to the strong correlation among type of particles, irregular factor and shape factor, irregular factor was rejected from the equations by discriminant analysis first. Type of particles used to be regarded as an important parameter[21], and turned out to have a limited value. The parameters, such as average number, positive rate, density and surface factor of particles were demonstrated to be valuable, and could reflect the characteristics of particles. The concordance rate was 79.57% for cancer group and 78.65% for adenoma group indicating this improved system is sensitive and very precise for quantifying the AgNOR dot count in cells and can provide a valuable objective measurement in differentiating benign from malignant tumors.

Relationship between PCNA and colorectal carcinoma

PCNA is a cell cycle related protein that is maximally elevated in late G1 and S-phase of proliferating cells and a key cycle regulator. It can be used as a marker of proliferation, and directly assessed using a thymidine analogue in suitably labeled pathological materials. Cellular proliferative activity has been accepted as a useful indicator of biologic aggressiveness in colorectal carcinoma[5-17]. PCNA immunohistochemistry could be used as a reliable marker of the proliferative compartment in both normal and neoplastic colonic mucosae[22]. The results revealed that all the parameters were significantly higher in adenoma and adenocarcinoma than in normal tissue, and the parameters in adenoma were higher than those in adenocarcinoma, indicating that multiplicative growth presented in G1 and S-phase when adenoma progressed to adenocarcinoma, and in other phase or in shock period while in adenocarcinoma. So the parameters of PCNA were descent in adenocarcinoma. Therefore we considered that the value of PCNA could distinguish benign from malignant lesions in the earlier stage of tumor.

Relationship among c-erbB-2, p53 and colorectal carcinoma

The c-erbB2 gene could be amplified in human adenocarcinomas, leading to elevated levels of expression of its encoded product, p185[11-23]. It has been shown that the accumulation of several alterations in p53 genes is most important for the conversion of adenoma to carcinoma. Critical genetic changes, including activation of oncogenes, mutation and deletion of tumor suppressor genes and disturbances in transcriptional regulatory sequences, might bring about aberrant expression of growth factors and their receptors in gastrointestinal carcinomas[10].

Mutations in the tumor suppressor gene p53 occur prevalently in a wide range of human tumors. Detection of a mutated p53 could provide useful information for the clinical management of colorectal neoplasms[24-29]. The p53 gene mutation and its subsequent over-expression in colorectal adenomas might therefore be a fundamental genetic event underlying the dysplasia and loss of proliferative control that are the characteristics of adenomas with a malignant potential[30]. Mutant p53 tumor suppressor gene and c-erbB-2 proto-oncogene were involved in human carcinogenesis, and detection of their protein product in human malignancies might influence the evolution of many neoplasms[31,32]. Therefore the aim of this study was to investigate the correlation of c-erbB-2, p53 with occurrence, progression of colorectal carcinoma and to determine the prognostic significance of oncogenes. The results showed that the values of c-erbB-2, p53 were increased in accordance with normal mucosa-adenoma-adenocarcinoma sequence (P<0.01), and the levels of c-erbB-2 were obviously increased in normal tissue and adenoma, suggesting that the occurrence of colorectal carcinoma was associated with activation of c-erbB-2, and c-erbB-2 could be considered as a more significant predictor of the occurrence of colorectal carcinoma. Meanwhile the value of total positive area and density of p53 were obviously increased in adenoma and carcinoma, indicating that p53 was a key oncogene in the progression of colorectal carcinoma. These results confirmed that p185 overexpression was associated with the early stages of colorectal cancer, whereas p53 was associated with more advanced stages[31].

Precancerous lesion of colorectal adenoma

The rate of carcinogenesis is associated with proliferation. To evaluate the proliferative degree of colorectal tumor with imaging analysis is helpful for the diagnosis of tumors. Our study of DNA contents, AgNOR, PCNA, p53, c-erbB-2 provided some useful prognostic information, their determination was useful for accurate evaluation of the prognosis.

The entity of carcinomatous change is a complicated process from quantitative to qualitative change. We studied the proliferative lesion with a mathematics model in order to provide the objective markers for accurate diagnosis. The result showed that DNA index, positive rates, density and aspect factor of AgNOR, densities of c-erbB-2, p53, shape factor and density of nucleus were relatively valuable. The result also showed that normal group was correctly diagnosed with the model (F>0.8). The 4 fault samples of papillary adenoma with grade 2-3 atypical hyperplasia by pathologic diagnosis were fault diagnosis of adenocarcinoma by the model (F<0.8). The 2 highly differentiated colorectal tumors were fault diagnosis of adenoma (F<0.8). Comprehensive evaluation showed the strong discriminating power of the model.

This study examined the value of PCNA. No parameter of PCNA was selected for the final model. As strong correlations were observed between PCNA and p53[33,34] we believed that the discriminating power of PCNA was hided by p53 due to the complex interface among the parameters. On the other hand, we doubted the value of PCNA in early cancer. However, some parameters of DNA, AgNOR, p53, cerbB-2 were selected for the model, and the diversity of tumor and occurrence and progression of colorectal tumor were related with multiple factors. Meanwhile, all the 4 indexes played a major role in the diagnosis of colorectal tumor, and inactivation of anti-oncogene and activation of oncogene were existed in the progression of colorectal malignancy.

Quantitative evaluation of some indices of colorectal tumor could provide reproducible data for its differential diagnosis. The discrimination model established can offer subjective parameters for distinguishing benign from malignant tumors.

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• BRIEF REPORTS •

Effect of antisense oligodeoxynucleotide of telomerase RNA on telomerase activity and cell apoptosis in human colon cancer

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Abstract

AIM: To explore the effect of antisense oligodeoxynucleotide (As-ODN) of telomerase RNA on telomerase activity and cell apoptosis in human colon cancer.

METHODS: As-ODN was transfected into SW480 cells by liposomal transfection reagent. Telomerase activity of SW480 cells was examined by telomeric repeat amplification protocol (TRAP) and enzyme-linked immunosorbent assay (ELISA). Apoptosis was analyzed by morphology and flow cytometry.

RESULTS: The telomerase activity in SW480 cells transfected with 1.0 μmol/L of As-ODN for 2-5 days, was significantly decreased in a time-dependent manner, and the cells underwent apoptosis. The missense ODN (Ms-ODN) and the control group transfected with SW480 cells did not show these changes.

CONCLUSION: As-ODN can specifically inhibit the telomerase activity of SW480 cells and induce apoptosis.

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INTRODUCTION

Colon cancer is one of the most common malignancies both in the world and in China[1]. More and more patients with early colon cancer can now be found due to the improvement in the diagnostic techniques. Although surgery and chemotherapy are effective on patients with localized tumors, the prognosis of patients with advanced or metastatic tumors is not ideal. As a result, it is absolutely necessary to explore a novel treatment modality, namely the gene therapy. Just like other kinds of cancer, colon cancer is now recognized as a genetic disease. Colon cancer cells contain many genetic alterations which accumulate as tumor develops. This makes it possible to treat cancer with gene therapy[2-3].

Telomerase is a ribonucleoprotein consisting of two components, RNA and protein. The RNA gene of telomerase is termed as human telomerase RNA (hTR). Two protein subunits have been found, which were named as human telomerase-associated protein (TEP1) and human telomerase catalytic subunit or human telomerase reverse transcriptase (hTERT)[4,5]. Telomerase activity in humans has been detected in germline and tumor tissues as well as in established cultured cell lines[6]. In normal somatic cells, the absence or low expression of telomerase is thought to result in progressive telomeric shortening with each cell division[7,8]. Therefore, it has been suggested that reactivation of telomerase is a critical step in tumorigenesis and that interference with the regulation of telomerase activity may serve as a basis for cancer therapy[9,10]. However, to our knowledge, whether antisense gene therapy directing against hTR is effective on colon cancer is unknown. We reported here the effect of antisense oligodeoxynucleotide of telomerase RNA on human colon cancer cell line, and investigated the potential value of telomerase as a target for antisense gene therapy of colon cancer.

MATERIALS AND METHODS

Cell culture

SW480 cells, a human colon cancer cell line, were provided by Department of Biology, Wuhan University, China, and maintained in RPMI 1640-10% fetal bovine serum supplemented with 1 mmol/L L-glutamine, 100 U/ml of penicillin plus 100 μg/ml of streptomycin at 37 ℃ under 5% CO_2.

Cell counting

SW480 cells were counted with 5 g/L of trypan blue staining.

Oligodeoxynucleotide synthesis

Two oligodeoxynucleotides were synthesized as described by Feng *et al* and Norton *et al*[11,12]. Antisense oligodeoxynucleotides (As-ODN) with the sequence 5' TAGGGTTAGACAA-3' , which can recognize the RNA template region of telomerase, and missense oligodeoxynucleotide (Ms-ODN) with the sequence 5' TGTAAGGAACTAG 3' were synthesized by Beijing SBS Biotechnology Engineering Company using the 391 DNA synthesizer. The synthesized oligodeoxynucleotides were subjected to electrophoresis (PAGE) and purified (300V, 1.5 h).

Transfection of oligodeoxynucleotides

Transfection of phosphorothiate oligodeoxynucleotides (ODNs) was carried out with liposomal transfection reagent DOSPER (Roche Diagnostic GmbH) according to the manufacturer' s protocol. Briefly, cells were plated onto 6-well plates and incubated until the cells reached 70-80% confluence. The DOSPER was diluted with serum-free medium the day before transfection. Then, the desired amount of ODNs was incubated for 15 minutes with diluted DOSPER. The ODNs/ DOSPER mixture (100 μl) was added dropwise into 900 μl of serum-free RPMI 1640. After incubated for 6 hours at 37 ℃, 1 ml of RPMI 1640 containing 20% FBS was added into each

Table 1 Inhibitory effect of telomerase activity by ODNs (mean±SD)

Groups	Active duration				
	24 h	48 h	72 h	96 h	120 h
As-ODN 10 μmol/L	0.872±0.194	0.406±0.232	0.386±0.146	0.307±0.203	0.289±0.213
Ms-ODN 10 μmol/L	1.063±0.249	1.285±0.179	0.959±0.273	0.109±0.243	1.247±0.178
Positive control	1.725±0.267	1.571±0.418	1.243±0.186	1.236±0.235	1.098±0.347
Negative control	0.349±0.092	0.312±0.076	0.283±0.089	0.063±0.072	0.057±0.023

well. Cells were harvested and analysed after 48, 72, 96 and 120 hours, respectively.

Telomerase activity assay

Telomerase activity was measured by polymerase chain reaction and enzyme linked immunosorbent assay (PCR-ELISA). Briefly, 2×10^6 cells were isolated, mixed with 200 μl of protein extraction buffer by vortes, and left on ice for 30 minutes. One hundred and seventy-five μl of supernatant was collected after centrifugation (16 000×g, 20 minutes, 4 ℃). PCR was performed in a system of 50 μl containing 25 μl of transfer reaction mixture, 2 μl protein extract and 2 μl primers, and 23 μl of nuclease-free water was added. The PCR condition was as the follows: at 25 ℃ for 30 minutes for primer elongation, at 94 ℃ for 5 minutes for telomerase inactivation. Thirty cycles of amplification were performed, each cycle was performed at 94 ℃ for 30 s for denaturation, at 50 ℃ for 30 s for annealing, and at 72 ℃ for 90 s for polymerization. Five μl of the amplified product and 20 μl of the denatured reagent were incubated at room temperature, 225 μl of hybridization buffer was then added and mixed, and 100 μl of them was distributed in the wells of a microtitering plate. After 2 hours of incubation (37 ℃, 300 rpm), 100 μl of anti-DIG-POD working solution was added and incubated for another 30 minutes, followed by the addition of 100 μl of TMB substrate solution, and 100 μl of stop reagent was added at last. The OD value in each well was read at the wave lengths 450 nm and 655 nm on a microtiter plate reader (Bio-RAD Model 550 microplate reader). The result of OD_{450} minus OD_{655} greater than 1.5 unit was judged as a positive control using a protein extract from immortalized telomerase-positive human embryonic kidney cells (293 cells). The negative control was considered as OD_{450} minus OD_{655} less than 0.2 unit by reading the protein extract pretreated with RNase A at 65 ℃ for 10 minutes. Telomerase activity was considered positive when the value of OD_{450} minus OD_{655} of a sample was at least 0.2 unit higher than that of the negative control, otherwise it was considered negative. Each sample was examined more than twice. The final value was presented as mean±SD after a statistical treatment by using *t* test.

Apoptotic features

To determine whether SW480 transfected with AS-ODN displayed an apoptotic morphology, the tranfected cells were observed under Olympus optical microscope and Hitach transmission electron microscope.

To determine the apoptotic rate and cell cycle distribution, the cells were fixed and stained by propidium iodide (PI, Sigma product), and analyzed by a FACSORT flow cytometer (Becton Dickinson). Briefly, cells were trypsinized, washed once in ice-cold PBS, and incubated with annexin-V-fluoroescein/PI, and then analyzed immediately by FACSORT flow cytometry. All data were analyzed using the Cell Quest software.

Statistical analysis

Results were expressed as mean±SD. Statistical analyses were carried out with the software package SPSS10.0. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Inhibitory effect of antisense hTR ODNs on telomerase activity

SW480 cells were transfected with As-ODN (1.0 μmol/L) and Ms-ODN (1.0 μmol/L), and collected at 24, 48, 72, 96 and 120 hours after transfection respectively. Telomerase activities were measured by TRAP-ELISA. Following results were found. The telomerase activity of SW480 cells transfected with As-ODN was greatly inhibited compared with that in the Ms-ODN. The telomerase activity of SW480 cells transfected with As-ODN at 72 and 96 hours after transfection was significantly lower than that both at 24 hours and in positive control as shown in Table 1. These findings suggested that this inhibitory action was sequence specific and in a time-dependent manner.

Effect of antisense hTR ODNs on induction of SW480 cell apoptosis

Cytologic morphological changes SW480, transferred with 1 μmol/L As-ODN for 3 days, cytologic morphology was observed under Olympus optical microscope and Hitach transmission electron microscope. It was found that cells rounded up off the plastids, exhibiting cytoplasmic blebbing, fragmentation and chromatin condensation, features of apoptosis. No apoptotic features (normal morphology) were observed in SW480 transfected with 10 μmol/L Ms-ODN (Figure 1).

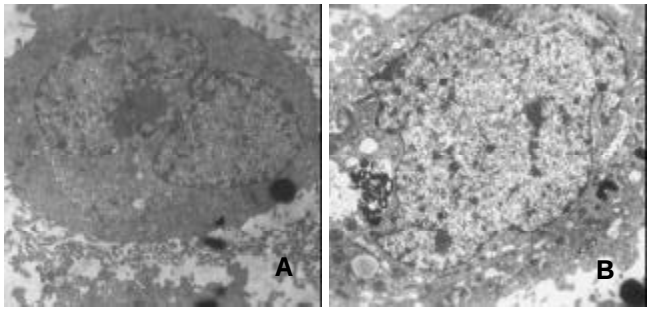

Figure 1 Morphplogic observation under transmission electron microscope. A: cells had no apoptotic features. B: cell rounded up off the plastic, exhibiting cytoplasmic blebbing, fragmentation and chromatin condensation, features of apoptosis.

Table 2 Effect of ODNs on induction of SW480 cell apoptosis (mean±SD)

Groups	Active duration		
	48 h	72 h	96 h
As-ODN 1 μmol/L	4.99±0.54	8.63±0.59	9.96±0.41
Ms-ODN 1 μmol/L	3.86±0.39	4.88±0.57	4.92±0.67
HRT blank	1.57±0.18	1.79±0.21	1.71±0.32

Detection of apoptotic cells

To determine the apoptotic rate, SW480 cells were transfected with 1 μmol/L As-ODN and Ms-ODN for 2 days. After permeabilization, the cells were stained with propidium iodide and analysed by flow cytometry. The apoptotic rate of SW480 cells transfected with As-ODN increased (4.99±0.54, 8.63±0.59,

and 9.96±0.41 at 48 h, 72 h and 96 h, respectively, $P<0.001$), but no significant changes of apoptosis were observed in SW480 cells transfected with 1 μmol/L Ms-ODN as shown in Table 2, indicating that this apoptotic induction was sequence specific and in a time-dependent manner.

DISCUSSION

Compared with normal somatic cells, cancer cells have an unlimited replicating capacity. This important characteristic of cancer, named immortality, has been gaining more and more attention, seeing that cancer cells might achieve cellular immortality through only a major pathway, the activation of telomerase[13]. Telomerase has been found to play an important role in carcinogenesis, thus becoming the basis of the widely held view of telomerase as a highly selective target for antisense gene therapy of cancer[14].

The RNA component of telomerase (hTR) was crucial to the telomerase activity[15-17]. Human cell lines expressing hTR mutated in the template region could generate the predictive mutant telomerase activity. Recent experiments have shown that antisense gene therapy directing against telomerase RNA component could effectively inhibit telomerase activity and induce apoptotic cell death in ovarian cancer, prostate cancer, bladder cancer, malignant gliomas and human breast epithelial cells[18-22]. However, whether such an anti-cancer effect can be obtained in human colon cancer is still unknown. Therefore, we examined the effect of antisense hTR oligodeoxynucleotide on human colon cancer cell line. As the results showed, our experiment clearly demonstrated that antisense-hTR oligodeoxynucleotide could significantly inhibit telomerase activity and induce apoptosis of human colon cancer cells, which was supported by the results obtained in our previous experiment[23]. All these findings provide the strong evidence that telomerase may be an ideal target for antisense gene therapy of human colon cancer.

Recently, it has been showed that telomerase activity was the dominant mechanism providing telomere maintenance to human immortalized cells. However, the exact mechanisms of how telomerase activity is regulated in tumour cells remain poorly understood. Some researchers have shown that telomerase activity correlated with the growth rate of immortal cells[24-26], whereas others found no significant association between telomerase activity and proliferation index in tissue specimens from breast carcinoma[27], gastric carcinoma[28], and Wilm' s tumour[29].

Inhibition of telomerase activity has been proposed as a potential method for the treatment of human malignancies. It is suggested that telomerase inhibition may serve as an effective tool for eliminating tumour cells that have short telomeres. Such tumours may provide reasonable targets for agents that inhibit telomerase. These experiments await the development of specific inhibitors for the components of telomerase complex.

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• BRIEF REPORTS •

Clinical and epidemiological data of patients with clonorchiasis

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Abstract

AIM: To study the clinical and epidemiological features of patients with clonorchiasis so as to provide scientific evidences for the diagnosis and prevention of clonorchiasis.

METHODS: Stools from 282 subjects suspected of having clonorchiasis were examined for helminth eggs with modified Kato's thick smear and sedimentation methods, and their sera were tested for HAV-DNA, HBV-DNA, HCV-RNA, HDV-RNA and HEV-RNA with polymerase chain reaction (PCR). Clinical symptoms of patients with clonorchiasis only were analyzed, and their blood samples were tested for circulating antigen (CAg) with Dot-ELISA, esoinophilic granulocyte count, and alanine aminotransferase (ALT). Meanwhile, they were asked to provide data of occupation, eating habit, hygienic habit and knowledge of clonorchiasis. In addition, the ecosystem of the environment in epidemic areas was surveyed.

RESULTS: Among the 282 patients, 61 (21.43%) were infected with *clonorchis sinensis* only, 97 (34.64%) were co-infected with *clonorchis sinensis* and other pathogens, 92 (32.86%) were infected with hepatitis virus only and 31 (11.07%) neither with *clonorchis sinensis* nor hepatitis virus. Among the 61 patients with clonorchiasis only, there were 14 (22.95%) subjects with discomfort over hepatic region or epigasfrium, 12 (19.67%) with general malaise or discomfort and inertia in total body, 6 (9.84%) with anorexia, indigestion and nausea, 4 (6.56%) with fever, dizziness and headache (6.56%), and 25 (40.98%) without any symptoms; sixty one (100%) with CAg (+), 98.33% (59/60) with eosinophilic granulocytes increased and 65.00% (39/60) with ALT increased. B-mode ultrasonography revealed 61 cases with dilated and thickened walls of intrahepatic bile duct, and blurred patchy echo acoustic image in liver. Twenty-six cases had stones in the bile duct, 39 cases had slightly enlarged liver with diffuse coarse spots in liver parenchyma. Twenty cases had enlarged gallbladder with thickened coarse wall and image of floating plagues, 9 cases had slightly enlarged spleen. By analysis of epidemiological data, we found that the ecologic environment was favorable for the epidemiology of clonorchiasis. Most patients with clonorchiasis were lack of knowledge about the disease. Their living environment, hygienic habits, eating habits and their occupations were the related factors that caused the prevalence of the disease.

CONCLUSION: The clinical symptoms of clonorchiasis are non-specific, and the main evidences for diagnosis of clonorchiasis should be provided by etiologic examination, B-mode ultrasonography and clinical history. The infection of *clonorchis sinensis* is related to occupations, bad eating habits and lack of knowledge about prevention of the disease.

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INTRODUCTION

Clonorchiasis, a food-borne infection by eating raw fish contaminated with *Clonorchis sinensis* metacercariae, is more frequently found in individuals along the great rivers and streams in areas of China, northern Vietnam, Korea and other Eastern Asia countries[1-8]. After oral infection, *C. sinensis* often engulfs the biliary epithelial tissues and blood cells in the intestine, and causes cholangitis with marked eosinophilic infiltration, biliary adenomatous hyperplasia, bile duct obstruction, and subsequently cholangiofibrosis[9-21]. In an effort to provide scientific evidences for diagnosis of this disease, clinical and epidemiological data of patients with clonorchiasis were analyzed in this study.

MATERIALS AND METHODS

Subjects

Two hundred and eighty-two individuals suspected of being infected with clonorchiasis were involved in this study, including 187 males and 95 females. Their age distribution was as follows: 29 aged 10-16 years, 182 aged 17-55 years and 71 aged 55-67 years.

Methods

Etiological examination Stools from 282 subjects were examined for helminth eggs with modified Kato' s thick smear and sedimentation methods, and sera of them were tested for HAV-DNA, HBV-DNA, HCV-RNA, HDV-RNA and HEV-RNA with polymerase chain reaction (PCR).

Analysis of clinical symptoms Clinical symptoms of the patients only with clonorchiasis diagnosed by microscopic fecal examinations were expressed as percentages according to the formula (the number of patients with some symptoms/ the total number of the patients with clonorchiasis)×100% and subsequently statistical analysis was conducted.

Laboratory examination Blood samples of patients only with clonorchiasis were detected for circulating antigen (CAg) with Dot-ELISA, counted for esoinophilic granulocytes, and tested for alanine aminotransferase (ALT). The number of esoinophilic granulocytes above 0.5×10^9/L was regarded as increased, and the value of alanine aminotransferase (ALT) above 30 u/L was judged as abnormal.

Examination by B-mode ultrasonography Abdominal part of the patients only with *C. sinensis* eggs in their stools was examined by B-mode ultrasonography.

Analysis of epidemiological data A questionnaire was given to the patients only with clonorchiasis, including occupation, eating habits, hygienic habits and knowledge about prevention

of clonorchiasis. In addition, we surveyed the ecosystem environments in the epidemic areas.

RESULTS

Etiological examination

Of the 282 individuals investigated, 21.43% (61/282) were confirmed to be infected with *C. sinensis* only by etiological examination, 34.64% (97/282) were infected with *C. sinensis* and other pathogens, 32.86%(92/282) were infected with hepatitis virus only, and 11.07% (31/282) with neither *C. sinensis* nor hepatitis virus (Table 1).

Table 1 Results of etiological examination

Types of pathogens	Case numbers	Percentages (%)
Infection of *C. sinensis* only	61	21.63
Infection of *C. sinensis* and other pathogens	97	34.40
Infection of *C. sinensis* and other helminth	32	11.35
Infection of *C. sinensis* and hepatitis virus	24	8.51
Infection of *C. sinensis*, other helminth and hepatitis virus	41	14.54
Infection of hepatitis virus only	92	32.62
Neither with C. sinensis nor hepatitis virus	32	11.35
Totally	282	100

Analysis of clinical manifestations

Among the 61 patients with clonorchiasis only, 14 (22.95%) subjects had discomfort over hepatic region or epigasfrium, 12 (19.67%) had general malaise or discomfort, 6 (9.84%) had anorexia, indigestion and nausea, 4 (6.56%) had fever, dizziness and headache, and 25 (40.98%) had not any symptoms.

Laboratory examination

The results of laboratory examination of the 61 patients with clonorchiasis only showed that the positive rates of CAg were 100%, eosinophilic granulocytes increased in 98.33%(59/60) patients and the values of ALT increased in 65.00%(39/60).

Examination by B-mode ultrasonography

In B-mode ultrasonography, 61 cases had dilated and thickened walls of intrahepatic bile ducts. Blurred patchy acoustic image was seen in liver. Twenty-six cases had calculi of intrahepatic ducts, 39 cases had slightly enlarged liver with diffuse coarse spots in liver parenchyma. Twenty cases had enlargement of gallbladder with thickened rough wall, floating plagues with postural variation were seen in the gallbladder. Nine cases had slightly enlarged spleen.

Analysis of epidemiological data

Occupational structure The constituent ratios of the occupation of 61 patients with clonorchiasis only were: fishermen 37.7%, fishmongers 9.84%, workers in processing fish products 16.39%, officials 19.67%, students 9.84% and others 6.56%.

Eating habits According to the results of questionnaires sent to 61 patients with clonorchiasis only, there were 26 subjects with habits of eating raw fishes and shrimps, 41 eating undercooked fish and shrimps, 48 with kitchen knives used in both raw and cooked food, 30 grasping food directly after contacting live fish and shrimps with unwashed hands, and 11 drinking unclean or unboiled crude water.

Knowledge about prevention of clonorchiasis All patients, did not have any knowledge about metacercaria of *C. sinensis* that might be in fish and shrimps of Huaihe River System. They had no idea about the possible infection with *C. sinensis* after ingestion of raw and undercook fish and shrimps. Even if infected with *C. sinensis*, symptoms such as epigastric pain, diarrhea, decreased appetite, dizziness, *etc.*, would occur.

Ecologic survey Among the sixty-one patients confirmed to be infected with *C. sinensis* only, 18 lived in villages along the Huaihe River, 19 lived near fishponds. We found a crowd of domestic animals coming and going on both banks of the river, such as chickens, ducks, dogs, pigs, *etc.*, and murine animals, hares and wild cats running in brushes and weeds near the river. In addition, wild ducks and birds, frolic king were looking for food on the river. Some mollusca, like *viviparidae*, *unionidar*, *bithyniidae*, *lymnaeidae*, and some fish and shrimps, such as *Pseudorasbora parva*, *Ctenopharyngodon idellus*, *Mylopharyngodon aethops*, *Cyprinus carpio*, *Lobstergrow* were found in waterweeds in the river.

About two thousand kinds of mollusca were collected and examined for cercaria of *C. sinensis*, and the positive rate was 0.25% (5/2000). When Ctenopharyngodon idellus was infected with cercaria, we could separate encysted metacercaria from its body. Similarly, the adult worms could be found from cats that were infected by encysted metacercaria. In addition, the positive rate of encysted metacercaria in fish and shrimps was 9.50%(19/200).

DISCUSSION

Clonorchiasis is endemic in East Asian countries and about seven million subjects were estimated to be infected with the fluke[22,23]. After oral infection with *C.sinensis* metacercariae, the organism excysts in the digestive tract and migrates up to the bile duct where it grows into adult worms. Infected humans excrete faeces containing *C.sinensis* eggs, and human infections could be diagnosed by microscopic fecal examination with demonstration of the characteristic eggs[24-26]. In the present study, we examined 282 subjects suspected of being infected with *C. sinensis* with methods of modified Kato' s thick smear and sedimentation, and 61 individuals were found to be infected with *C.sinensis* only, while 97 were co-infected with *C.sinensis* and other pathogens, 92 were infected with hepatitis virus only, 31 had neither *C. sinensis* nor hepatitis virus. Etiological examination is a major method in the differential diagnosis of clonorchiasis and other diseases of the liver and biliary tract.

The prevalence of clinical symptoms was determined by the fluke quantity, frequency of infection and the hosts immunities. Subjects with mild infection of *C.sinensis*, often had symptoms or only some epigastric discomfort, anorexia, dyspepsia, *etc*. Subjects with moderate infection often had inertia, lassitude, dyspepsia, abdominal pain and other mild complaints, all of which could also be obviously observed in the subjects with severe infection and cirrhosis of liver. Portal hypertension and ascitis might finally occur[27-32]. The outcome of this study showed there were 40.98% of patients without any symptoms and others with symptoms of epigastric discomfort, general malaise, inertia, anorexia, dyspepsia, nausea, fever, dizziness, headache, *etc*. had no specificity. Differential diagnosis should include schistosomiasis Japonica, fascioliasis and fasciolopsiasis.

During the development of *C. sinensis* in human body, CAg was produced and released into circulation. Generally, CAg might be detected in sera of the subjects three days after infection[33,34]. For this reason, detection of CAg may be used to examine whether live worms exist in human body or not, and for check up of treatment effects. By Dot-ELISA, all of the 61 *C. sinensis* egg-positive individuals were positive, although 40.98% of them had no symptoms. So detection of CAg may provide an evidence for diagnosis of clonorchiasis. In addition, results from this study showed that eosinophilic granulocytes were increased in 98.33% patients, ALT was increased in 65% patients and image changes were found in

liver with B-mode ultrasonography in all patients. These are all helpful in the diagnosis of clonorchiasis.

Both natural and social factors affect transmission of *C. sinensis*. We investigated the ecologic system in the Huaihe River on the spot and saw various kinds of mollusca crawling on water plants and some fish and shrimps swimming in the river, all of which are intermediate hosts of *C. sinensis*. Near the river, a large number of domestic animals, livestock, murine animals, hares and wild cats are reservoir hosts. Moreover, the villagers have got accustomed to feeding fish with their own stools and domestic animals with raw fish and shrimps, which help *C. sinensis* to be transmitted from one host to another. On both banks of the river, simple toilets and pigsties are arranged everywhere, and the villagers brush chamber pots in the river without scruple. Terribly, they live on water untreated from the river. All of these natural environments and human activities contribute to overflowing of *C. sinensis* in this area.

The incidence of clonorchiasis was shown in this study to vary greatly and was related to occupations, and was higher in fishermen, fishmongers, workers in processing fish products, officials than in those with other occupations. Because of bad working and eating habits, they have chances to be infected with encysted metacercaria. Therefore, knowledge about prevention of clonorchiasis should be strengthened in these populations to correct their bad eating and health habits and to reinforce their protection consciousness.

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• BRIEF REPORTS •

Effects of Cd^{+2}, Cu^{+2}, Ba^{+2} and Co^{+2} ions on *Entamoeba histolytica* cysts

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Abstract

AIM: The effects of cobalt, copper, cadmium and barium ions on the cysts of *Entamoeba histolytica* (*E. histolytica*), an amebic dysentery agent, cultured in Robinson medium were investigated.

METHODS: *E. histolytica* cysts and trophozoites isolated from a patient with amebiasis were cultivated in the medium, incubated at 37 ℃ for a period of 4 days and 40×10^4/ml amebic cysts were then transferred to a fresh medium. At the second stage, 0.05, 0.1 and 0.2 mM of selected metal ions were added to the medium, and the effects of these ions on parasitic reproduction compared with the control group were observed.

RESULTS: It was determined that the number of living parasites in all the groups containing metal ions decreased significantly starting from 30 minutes ($P<0.01$). $CuCl_2$ showed the highest lethal effect on *E. histolytica* cysts, whereas the lowest lethal effect was observed with $CoCl_2$. It was also seen that the number of living cells was decreased as the ion concentration and exposure time were increased, and that there were no living parasites in the medium at the end of 24 h ($P<0.01$).

CONCLUSION: It may be stated that the effect of ever-increasing contamination of the environment with metal waste materials on parasites should be investigated further.

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INTRODUCTION

Amebiasis, which infects nearly 10% of the world population and is responsible for mortality and morbidity in developing countries in particular is caused by a protozoon, *Entamoeba histolytica* (*E.histolytica*)[1]. The form of the agent infecting humans is the 4 nuclei cysts, shed into the environment with feces. The cysts, which can survive in the environment for a long time, can carry on their life cycle with fecal-oral contamination[2,3].

Like many other protozoa, this particular parasite develops complex metabolic structures under different environmental conditions[4]. Reproduction of *E. histolytica* in *in vitro* culture provides a better understanding of its biological characteristics, its pathogenesis and adaptation to environmental conditions[2]. The metabolism of the parasite in a medium environment varies according to the age of the culture, its oxidation-reduction potential, as well as the composition, the temperature of the medium and the accompanying bacteria[5,6]. It was reported that *E. histolytica* cysts reproduced under axenic conditions had cyst walls, which were different from, and weaker than those found under natural conditions[7]. For this reason, it is believed that it would be more appropriate to use xenic media as they reflect the morphological structure and living conditions of the parasite[8]. One of the most commonly and successfully used xenic media is the one defined by Robinson[9].

In these complex biochemical reactions of microorganisms, certain metal cations play an important role as trace elements[10]. However, some of them are not favored by the ecosystems of living cells, and doses which accumulate in the cell in time (metal ions) do not agree with life[11,12]. Heavy metal ions in particular bind to sulphydril (-SH) groups and form complexes, leading to toxic effects on living cells. This situation can be determined by the blurring of the reproduction media of microorganisms especially in the experimental studies carried out with *in vitro* cultures[13]. Setting out from this point, knowing the reaction of *E. histolytica* cysts shed into the environment with feces to the metal ions found in the area for various reasons will especially help understand the impact of the contamination by these ions on the parasite population[14,15].

In this study, we investigated the effects of cadmium (Cd^{+2}), copper (Cu^{+2}), barium (Ba^{+2}) and cobalt (Co^{+2}) ions on *E. histolytica* clinical isolate xenically produced in Robinson medium. In the light of the data we obtained, we also investigated what kinds of effects these metal ions produced with different intracellular concentrations in the parasite depended on time, and whether there were any differences among these ions with respect to their tendency to produce such effects.

MATERIALS AND METHODS

Clinical isolate and culture

E. histolytica fresh clinical isolate was obtained from a patient with amebiasis who came to the laboratory of Dokuz Eylul University Medical Faculty Department of Parasitology. As a result of an examination of the stool samples of the patient by wet mount and Lugol's iodine and trichrome staining methods, *E. histolytica* cysts and trophozoites were determined and the specimen was cultivated in Robinson medium[9]. The tubes were incubated at 37 ℃ for 4 days without subculturing. The cysts were then washed with sterile distilled water at pH 7 and counted with a haemocytometer. The 40×10^4/ml amebae cysts were transferred to a fresh medium under the same conditions and ions prepared at different molarites were immidiately added to the tubes.

Cd^{+2}, Cu^{+2}, Ba^{+2} and Co^{+2} ion concentrations

Cd^{+2} [Cadmium chloride ($CdCl_2.5/2H_2O$) (Merck; NJ, USA)],

Cu^{+2}[Cupric chloride ($CuCl_2.2H_2O$) (Merck; NJ, USA)], Ba^{+2} [Bariumchloride ($BaCl_2.2H_2O$) (Riedel-de Haën; Seelze, Germany)] and Co^{+2} [Cobalt chloride ($CoCl_2.6H_2O$) (Carlo Erba Reagenti; Rodano (Mi), Italy)] ions prepared at 3 different molarities, namely 0.05, 0.1 and 0.2 mM, were added to the study group[15]. The remaining tubes, on the other hand, were kept as control group without addition of metal ions, and also serum was added to the control tubes. Three tubes were prepared for each ion molarity and also for control group. Each determination was performed in triplicate.

Parasite counting

All the medium tubes were incubated at 37 ℃. After 0.5, 1, 2, 3, 5, 7 and 24 h, the tubes were stirred for 5-10 seconds to achieve a homogeneous distribution and a drop of medium specimen was taken with the help of a Pasteur pipet. A drop of 1% eosin saline solution was added to the specimen. Unstained cystic structures were considered to be alive, whereas those which let pink-red stains in were regarded to be dead. Living cysts were counted with a haemocytometer and the measurements were recorded.

Statistical analysis

All the groups in this study were evaluated by means of GLM-repeated measures, post hoc Tukey in the SPSS for windows 8.0 statistical program and Mann-Whitney *U* test within themselves. $P<0.05$ was considered significant.

RESULTS

The number of living amebae per ml determined in each tube at 0.5, 1, 2, 3, 5, 7 and 24 h after metal ions and serum were added to the medium environment, is shown in Figure 1. The numbers of living cells were significantly decreased in all the groups compared to control group ($P<0.01$). It was determined that the most effective metal ion was Cu^{+2} ($P<0.01$), in contrast the least effective metal ion was Co^{+2}. In all the groups, the number of living cells was decreased as the concentration of the metal ions was increased ($P<0.01$).

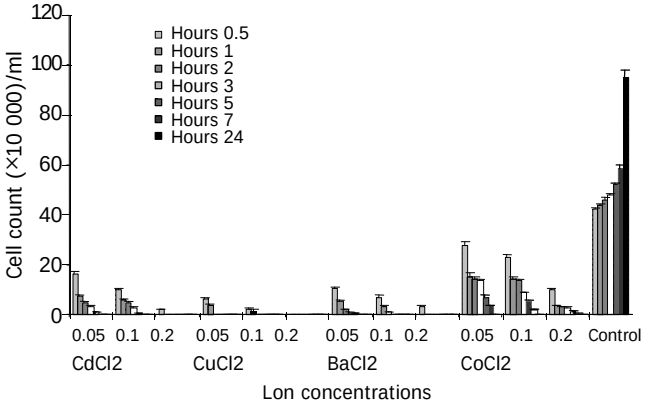

Figure 1 Effect of different concentrations of metal ions on *E. histolytica* cysts. X-axis: the molarities of ion concentration. Y-axis: The alteration of living parasite counts dependent on time and ion molarities.

Judging from the facts that they let 1% eosin-saline solution in themselves and that their cell walls degenerated and degranulated, it was found that the amebae cysts were dead after 24 h at each of the 3 concentrations of all the metal ions. It was also observed that heavy metals with larger atomic numbers affected the functions of intracellular physiologic cations, forming metal complexes. Such a complex formed with Cu^{+2} was seen at any concentration for Cd^{+2}, however, the formation of complexes was started from 0.1 mM. The formation of this complex was indicated by the blurring of the medium.

As for the effect of Co^{+2}, it was found that *E. histolytica* cysts survived until the end of the 7 th h at 0.05 and 0.1 mM concentrations, whereas this was not the case for the other metal ions. Similarly, it was observed that at 0.2 mM of Co^{+2} living amebae cysts were found at the end of the first hour only ($P<0.01$).

It was also observed that most of the cysts in the control group tubes, cultivated in a fresh medium, transformed into trophozoites and started to reproduce.

DISCUSSION

To be aware of the effects of toxic metals on living things is of great importance, especially in view of the ever-increasing environmental pollution. A number of researches about the effects of metal ions on the physiologic functions of various microorganisms are available[16-18].

It is believed that the toxic effect of Cd^{+2}, an important heavy metal, might be associated with the denaturation of proteins as a result of its binding to the thiol groups in living cells or with membrane damage stemming from its interaction with Ca^{+2}[17]. It was reported that the toxic effect of Cd^{+2} on the culture medium of *Trypanosoma brucei brucei* was at 0.045 mM level[19]. We determined in this study that when we used 0.05 mM Cd^{+2} as the lowest concentration, the lethal effect leading to the death of living *E.histolytica* cysts started at 0.5 h and was increased time-dependently. We also observed that, Cd^{+2} made the most complexes in the medium starting from 0.1 mM. In this context, the result we obtained was comparable with relevant literature data[19].

Cu^{+2}, however, can interact with radicals, oxygen in particular. These radicals also cause Cu^{+2} to become toxic. A study carried out on pseudomonas by means of spectroscopy, reported that Cu^{+2} caused greater damage than Co^{+2} did[20]. In the present study, we discovered that the lethal effect of metal ions at different concentrations was dependent on time rather than the extent of damage and found that Cu^{+2} ions were more effective than Co^{+2}. Another study in which the toxic effect of cadmium acetate and copper sulphate salts on the mouse trachea culture was investigated, reported that the effect of cadmium acetate was shown after 35 minutes, while that of copper sulphate came about after 85 minutes although equal amounts were added to the medium, accordingly the effect of cadmium acetate on the cell was greater[21]. As a different view, in a study where the cytotoxic effect of cadmium and copper was investigated in the culture of *Merceneria mercenaria*, the cytotoxicity concentration of Cd^{+2} was started at 0.1-1.5 mM and that of Cu^{+2} at 0.01-0.1 mM[22]. In our study, however, we investigated the effect of 0.05, 0.1 and 0.2 mM metal ions on *E. histolytica* cysts in a culture environment. When the effect of both metal ions was investigated starting as early as 0.5 h, we found the toxic effect of Cu^{+2} on amebae cysts to be greater. Cu^{+2} ions also bound to various compounds and formed complexes. These complexes were determined by the blurring of the medium environment and led to toxic effects on *E.histolytica* cysts at each molarity.

On the other hand, Co^{+2} enters the composition of B_{12}, the main cofactor. This element accumulates in the core structure of microorganism cells. Ermolli *et al*. reported that 0.475 mM Co^{+2} produced a toxic effect on HaCaT human keratinocytes and that this effect could only be observed after 4 h[23]. It was found in our study however, the lethal effect of Co^{+2} on *E. histolytica* was presented at lower concentrations such as 0.05 mM and was increased dose-dependently. It was also observed that amebae cysts maintained their vitality, though at a minimal level, at all the three concentrations at the end of

the 7th h and that no living parasite remained in the culture medium at the end of the 24 th h. In the light of these data, we believe that Co^{+2} at low concentrations has a slowly developing lethal effect on *E. histolytica* cysts.

Certain studies aiming at determining the effects of Ba^{+2} have been carried out in cell cultures[24,25]. Borella *et al*. reported that addition of $10^{(-4)}$ -$10^{(-6)}$ mol/L Ba^{+2} to the culture medium did not have any effect on reproduction[24]. When we investigated the effect of 0.05, 0.1 and 0.2 mM Ba^{+2} on *E. histolytica* cysts in Robinson medium, we found that the lethal effect was dependent on both the dose and time.

$CdCl_2$, $CuCl_2$, $BaCl_2$ and $CoCl_2$ were used for evaluating the effects of these metal ions on the growth of *E. histolytica* cysts. As it was shown the only anion was chlor, all differed in the metal cations.

It would not be surprising to predict that investigating the effects of various factors causing environmental pollution on the life cycle of parasites, which have an important place among microorganisms, would be one of the objectives of future scientific studies. Accordingly, when the effect of Cd^{+2}, Cu^{+2}, Ba^{+2} and Co^{+2} ions on *E. histolytica* was investigated, we determined that these metal ions, led by Cu^{+2}, prepared at concentrations of 0.05, 0.1 and 0.2 mM had a lethal effect on the parasite. Considering the fact that the life cycle of *E. histolytica* depends on mature cysts with 4 nuclei shed into the environment with feces, this particular result may have a considerable significance in terms of the ecological balance of the environment, which is being contaminated by various metal waste materials.

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• BRIEF REPORTS •

Frequency of toxoplasmosis in patients with cirrhosis

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Abstract

AIM: It is known that toxoplasmosis rarely leads to various liver pathologies, most common of which is granulomatose hepatitis in patients having normal immune systems. Patients who have cirrhosis of the liver are subject to a variety of cellular as well as humoral immunity disorders. Therefore, it may be considered that toxoplasmosis can cause more frequent and more severe diseases in patients with cirrhosis and is capable of changing the course of the disease. The aim of this study was to investigate the frequency of toxoplasmosis in patients with cirrhosis.

METHODS: Serum samples were taken from 108 patients with cirrhosis under observation in the Hepatology Polyclinic of the Gastroenterology Clinic, and a control group made up of 50 healthy blood donors. IFAT and ELISA methods were used to investigate the IgG and IgM antibodies, which had developed from these sera.

RESULTS: Toxoplasma IgG and IgM antibody positivity was found in 74 (68.5%) of the 108 cirrhotic patients and 24 (48%) of the 50 people in the control group. The difference between them was significant ($P<0.05$).

CONCLUSION: In conclusion, it was found that the toxoplasma sero-prevalence in the cirrhotic patients in this study was higher. Cirrhotic patients are likely to form a toxoplasma risk group. More detailed studies are needed on this subject.

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INTRODUCTION

Toxoplasmosis is a protozoan disease that infects 35-40% of the adult population of the world and demonstrates varying clinical manifestations. Its active agent is *Toxoplasmosis gondii* (*T. gondii*). In man tissue parasitism during the proliferative phase may occur without signs of symptoms. It may lead to a transient illness characterized by lymadenopathy, fever and fatigue, or a severe disease. Severe manifestations of the disease most commonly occur in patients with impaired immunity[1].

In most countries, seroprevalence of toxoplasma ranges between 20% and 60%. The prevalence is quite low in extremely dry and cold regions. It has been reported that the prevalence is rather high in warm and humid areas[2].

Cats, small mammals and birds take place in the usual life cycle of *T. gondii* in nature. Humans join this chain as a result of their close relationship with cats. Toxoplasmosis is never encountered in the small Pacific islands where there are no cats. In the group investigated for toxoplasmosis, the prevalence in Turkey ranged between 44% and 55%[3,4].

Toxoplasmosis may rarely cause various liver pathologies due to granulomatose hepatitis in patients with normal immune systems[1,5-8].

Patients with cirrhosis of the liver demonstrate various cellular and humoral immunity disorders[9-12]. For this reason, it may be thought that toxoplasmosis may lead to more frequent and more severe diseases in patients with cirrhosis and change the course of the disease.

What was investigated in this study was the frequency of *T. gondii* antibodies in the cases of cirrhosis associated with various reasons.

MATERIALS AND METHODS

One hundred and eight patients with cirrhosis from the Hepatology Polyclinic of the Gastroenterology Clinic, and a control group comprising 50 healthy blood donors of similar age and sex were taken in the study. Serum samples were taken from the patients and control group and kept at -20 ℃ until toxoplasma serological tests were performed.

IgM and IgG antibodies from the sera were investigated by IFAT and ELISA methods.

ELISA method

Dissolved antigen was prepared based upon literature data provided by Herlow *et al*, Naot *et al*[13,14]. Serum samples were diluted up to 1/64, 1/256, 1/1 024, 1/4 096 to determine IgM antibodies and up to 1/256, 1/1 024, 1/4 096, 1/8 000, 1/32 000 to determine IgG antibodies. The sera were read at a 405λ wavelength ELISA reader (Titertek II). The mean absorbance values of negative controls were added to the 2 standard deviation values of these absorbance values. Those above the cut-off value obtained were accepted as positive and compared with the values expressed by the control sera to assess the suspected sera. For IgG 1/1 024 and above and for IgM 1/256 and above were accepted as significant titers with regard to active disease[15].

IFAT method

Particle antigen was prepared according to data from Garin *et al*, Remington *et al*[16,17]. Serum samples were diluted and assessed semiquantatively. The dilution of the sera within the scope of the study was 1/16, 1/64, 1/128, 1/256, 1/512, 1/1 024, 1/4 096 for both IgG and IgM. The results obtained were assessed by a fluorescence microscope (Nikon) at 490 nm stimulation, 510 nm barrier filter wavelength and 20×10 magnification. For IgG 1/256 and above and for IgM 1/16 and above were accepted as significant titers with regard to

Table 1 Cirrhosis etiology of 108 patients

	Number of patients	%
Hepatitis B	37	34.3
Hepatitis C	27	25
Autoimmüne hepatitis	5	4.6
Alcoholic cirrhosis	18	16.7
Primary biliary cirrhosis	12	11.1
With unknown etiology	9	8.3
Total	108	

Table 2 Toxoplasma IgG and IgM positivity of patients and control groups

	Number of patients	Sex W / M	Age	IFAT and ELISA IgG(+)	IFAT and ELISA IgM(+)	Active disease significant with respect to	
						Ig G(+)	IgM(+)
Cirrhosis	108	38 / 70	51.5±10.2	74 (%68.5)[a]	2	31(%28.7)[a]	2
Control	50	19 / 31	40±6.7	24 (%48)	-	4 (%8)	-

[a]$P<0.05$.

active disease[15].

Comparisons between the cirrhotic patients and the control group pertaining to antibody positivity and sex were performed according to Fisher exact age distribution *t* test.

RESULTS

Cirrhosis etiology in patients is shown in Table 1. The cirrhotic patients and the control group demonstrated similar sex and age distributions (Table 2). Toxoplasma IgG and IgM antibody positivity was determined in 74 (68.5%) of the 108 cirrhotic patients and 24 (48%) of the 50 individuals in the control group. The difference was significant ($P<0.05$). Significant titers were found with respect to active disease (IgG 1/1 024 and above, IgM 1/256 and above for ELISA, and IgG1/256 and above, IgM 1/16 and above for IFAT) were found in 31 (28.7%) of the cirrhotic patients and 4 (8%) of the control group. The difference was significant (Table 2).

DISCUSSION

Toxoplasmosis is a protozoan disease that is widespread all over the world and demonstrates varying clinical manifestations. Determination of its incidence in various risk groups in the society and establishment of these risk groups play a significant role in taking the necessary precautions against this disease.

In this study toxoplasma IFAT and ELISA antibody positivity was significantly higher in cirrhotic patients. Besides, the significant titers were found to be higher with regard to active disease.

Toxoplasmosis can be frequently found in the general population all over the world. It has been reported that it was encountered at a higher rate in warm and humid regions compared to cold and dry places[2]. No sero-epidemiologic studies that would properly demonstrate the toxoplasmosis prevalence in the whole population in Turkey have been reported so far. The studies carried out merely reflect the results of those that have been brought to and evaluated in various laboratories with suspicion of toxoplasmosis. In a study carried out in Elazıg, a region of Turkey that is comparatively underdeveloped from the socio-economic point of view, Asci *et al*. found toxoplasma antibodies in 55% of 1 641 serum samples[3]. In a study covering the Aegean region between 1991-1995, Altintas *et al*. determined toxoplasma seropositivity in 4 651 (49.4%) of 9 410 individuals in their study[18]. Sutcu *et al*. found the toxoplasma IgM positivity was 10% and IgG positivity was 44% in Konya province between 1993 and 1997[4]. The seroposivity rate in Turkey generally varies between 44% and 55%. These values are quite close to the rates we have determined in our control group (48%), but lower than those in cirrhotic patients (68.5%). No other study has investigated the toxoplasma antibody frequency in cirrhotic patients. This study is probably the first one investigating the toxoplasma seroprevalence in cirrhotic patients.

The reasons why both the antibody positivity and titers were significant with regard to active disease are not known. Could toxoplasma, known to cause partial damage to the liver, have a role in the onset and clinical course of cirrhosis?

This study did not contain any research into the activity of toxoplasma. Nevertheless, the fact that antibody titers are higher in cirrhotic patients leads one to think that these people might have an active disease. We are also planning another study to determine whether active disease develops in cirrhotic patients by monitoring the changes in the long-term toxoplasma titers.

To sum up, the toxoplasma sero-prevalence in cirrhotic patients in our study was found to be higher. Cirrhotic patients may well form a risk group for toxoplasma. More detailed studies need to be carried out on this particular subject.

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• BRIEF REPORTS •

Probiotics inhibit TNF-a-induced interleukin-8 secretion of HT29 cells

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Abstract

AIM: To study the effect of probiotics on interleukin-8 secretion in intestinal epithelia when stimulated by proinflammatory cytokines.

METHODS: Colonic adenocarcinoma HT29 cells were cultured and divided into four groups: control, TNF-α (group T in short), bifidobacterium (group B), lactobacillus (group L). B. Longum and L. bulgaricus were suspended in culture medium with a concentration of 1×10^8 cfu/ml and added into 24 wells respectively. One hour later TNF-α (10 ng/ml) was added into each well of groups T, B, L. The supernatants were collected and measured for IL-8 after 3 hours, nuclear factor-κB (NF-κB) p65 was also examined by Western blotting.

RESULTS: There was less interleukin-8 secretion in HT29 cells when preincubated with B. Longum or L. bulgaricus compared with group T. Less p65 appeared in nuclei in groups B and L compared with group T, as detected by Western blot.

CONCLUSION: Probiotics can suppress interleukin-8 secretion in intestinal epithelia when stimulated by proinflammatory cytokines, which is most likely mediated by NF-κB.

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INTRODUCTION

Intestinal epithelium is an important factor of gut mucosal barrier, and participates in innate immunity. Intestinal epithelia are capable of releasing some proinflammatory cytokines such as IL-8 when stimulated by cytokines like TNF-α, and can response to enteric pathogens and release some proinflammatory cytokines which in turn direct the movement of inflammatory cells of the lamina propria[1]. Probiotics, including bifidobacterium, lactobacillus play an essential role in the completeness of intestinal mucosa barrier. For example, some probiotic strains could modulate intestinal mucosal immune response, some could play protective roles by inhibiting the adhesion of pathogenic bacteria to intestinal epithelia. The present study was to investigate the effect of probiotics on IL-8 secretion of intestinal epithelium induced by TNF-α, and its possible mechanism.

MATERIALS AND METHODS

Reagents

rh TNF-α was obtained from Pepro Tech Ec Ltd,UK. Rabbit anti-human NF-κB p65 polyclonal antibody, peroxidase-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology Inc.,USA. Human IL-8 ELISA kit was supplied by Jingmei Biotech Co. Ltd., China. BHI-agar was provided by Oxoid Co.,UK. TMB membrane peroxidase substrate system was provided by KPL Inc., USA.

Methods

Bacteria Bifidobacterium longum and lactobacillus bulgaricus LB10 were provided by the Department of Microbiology, Huaxi School of Stomatology, Si chuan University. The strains were grown at 37 ℃ in static, nonaerated BHI-agar to reach the mid-log phase. Bacteria were harvested by centrifugation at 2 500 g for 15 min at 20 ℃. After two washes in sterile PBS pH 7.4, at 25 ℃, the bacteria were resuspended in PBS. Cell counts in the bacteria suspension were estimated by optical density at 600 nm absorbance (BioMerieux, Germany). Then the bacteria were added to the cell culture wells at appropriate dilution to reach a final concentration of 10^8 cfu/ml of medium.

Cells and bacteria coculture HT29 cells were grown in RPMI1640 with 10% fetal calf serum, and divided into four groups: control, TNF-α (group T in short), bifidobacterium (group B), lactobacillus (group L). When grown to confluence in single layer, cells were washed three times with PBS pH 7.4, to remove culture medium and nonadherent cells. The bacteria in culture medium were transferred into individual wells respectively. TNF-α (10 ng/ml) was added into each well of groups T, B, L 1 hour later. The supernatants were collected and centrifuged for measurement of IL-8 after 3 hours.

IL-8 enzyme-linked immunosorbent assays IL-8 enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's instructions. In short, polyclonal goat anti-human IL-8 antibodies were used as capturing antibodies, biotinylated polyclonal rabbit anti-human IL-8 antibodies as detecting antibodies. Streptavidin-HRP and TMBS were added as color indicator. Plates were read at 450 nm of wavelength right after color reaction was stopped with acid. All procedures were performed at room temperature.

Assessment of NF-kB activation by Western blotting Nuclear extracts were prepared according to the protocol described by Schreiber, *et al*[2]. Nuclear proteins were separated by SDS-polyacrylamide mini-gel electrophoresis at a constant current of 30 mA for 150 min, then transferred to nitrocellulose membranes and stained with ponceau S to verify equal protein loading. Membranes were blocked in 5% milk in Tris-buffered saline for 4 h at 4 ℃, incubated overnight at 4 ℃ with anti-human p65 polyclonal antibody (at a dilution of 1:1 000) followed by 1 h incubation at room temperature with peroxidase-conjugated goat anti-rabbit IgG (1:2 000). Finally,

the membranes were incubated in TMB membrane peroxidase substrate solution.

RESULTS

IL-8 secretion

Concentrations of IL-8 in supernatants of each group are shown in Figure 1. The concentration of IL-8 in control was only 172.2±42.1 ng/L. When stimulated by TNF-α, HT29 cells secreted a large number of IL-8, and the concentration of IL-8 in group T was 639.5±62.3 ng/L. However, when preincubated with B. Longum or L. bulgaricus, HT29 cells produced less IL-8, compared with group T, and the concentrations of IL-8 were 461.8±76.7 ng/L, 515.4±55.4 ng/L in groups B and L, respectively.

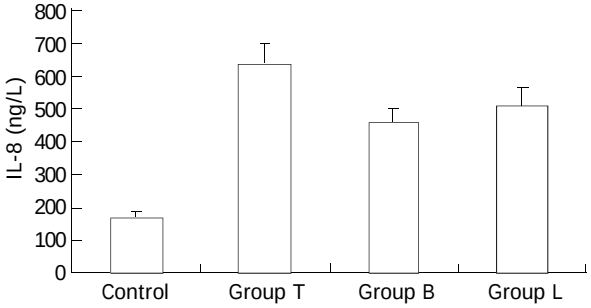

Figure 1 Concentrations of IL-8 in each group (mean±SD). Natural interleukin-8 expression was seldom found in HT29 cells of control group. When stimulated by TNF-α (10 ng / ml), HT29 cells secreted a large number of IL-8, and the concentrations IL-8 in groups T, B and L were significantly increased than that in control (P<0.001). However, there was some difference in the concentration of IL-8 among groups T, B and L. There was less interleukin-8 secretion in HT29 cells when preincubated with B. Longum or L. bulgaricus in groups B and L compared with group T (P=0.002, 0.01, respectively), but there was no difference in concentration of IL-8 between groups B and L (P=0.068).

Detection of nuclear p65

There was little NF-κB p65 in nuclei of normal epithelia without any stimuli, and it was very difficult to detect p65 in those cells by Western blotting. The epithelia expressed high levels of nuclear NF-κB p65 when stimulated by TNF-α. Decreased expression of nuclear NF-κB p65 appeared in cells of groups B and L, in contrast to group T, as Western blotting showed (Figure 2).

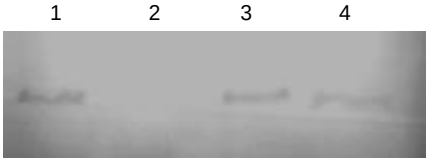

Figure 2 Expression of nuclear NF-κB p65 in each group. Line 1: group T, Line 2: control, Line 3: group L, Line 4: group B.

DISCUSSION

Intestinal epithelia constitute mucosal barrier of the bowel, and participate in inflammatory or immune responses in gut[3,4]. In some gastrointestinal infectious and inflammatory conditions, such as inflammatory bowel disease (IBD), acute gastroenteritis, inflammatory cells including monocytes, lymphocytes, were activated and accumulated in lamina propria. The cells secrete excessive inflammatory products, such as TH1 type cytokines, chemokines and a lot of active oxides. Overproduction of cytokines could affect the biological action of epithelial cells. For instance, TNF-α could induce epithelial cells to secrete IL-8, and express membrane Toll-like receptor 4 (TLR4) excessively[5,6]. TLR4 could enable intestinal epithelia hyperreactive in response to lipopolysaccharides (LPS), the component of bacteria walls, and IL-8 had leukocytes chemotactic and stimulatory properties[7]. As more inflammatory cells infiltrate, the inflammatory reaction is therefore amplified.

The normal flora of human gastrointestinal tract contains diverse populations of bacteria which play an essential role in the development of gut mucosal barrier and innate immunity. Some intestinal microflora could exert a protective role against pathogens[8]. Aberrance of gut microflora has been reported in IBD and acute gastroenteritis[9,10]. The aberrant microflora dysregulates mucosal immune reaction. Invasion of some virulent strains into epithelia could break down the integrity of intestinal mucosa, and induce inflammatory cell infiltration[11]. Some researchers found that manipulating the normal intestinal flora using probiotics had a beneficial effect on health by altering the microbial environment, and some components of the flora could down-regulate inflammation when supplemented to patients with gastrointestinal diseases[12,13]. Some studies have been undergoing to explore the possible mechanisms of probiotic action on gut epithelium and mucosal immune system.

In order to imitate the inflammatory condition of gut *in vitro*, we used TNF-α to stimulate human colonic adenocarcinoma HT29 cells, which has basically the same biological properties as normal colonic epithelia. As some probiotic strains could adhere to human intestinal cell surface[14,15], two probiotic strains, B.longum and L.bulgaricus, inhibited the secretion of IL-8 in HT29 cells when stimulated with TNF-α one hour after coculture with the two probiotic strains. It indicated that the strains could trigger anti-inflammatory pathways within the gut epithelium. The epithelia attached to the strains showed immune hyporeaction to TNF-α, and produced less IL-8. Because transcriptional control of IL-8 was mediated by transcription factor NF-κB[15], we investigated the expression of nuclear NF-κB p65 in HT29 cells. There was little NF-κB p65 in nuclei of normal epithelia without any stimuli. TNF-α highly up-regulated the expression levels of nuclear NF-κB p65. However, the epithelia pre-cocultured with two probiotic strains before TNF-α treatment showed a decreased expression of nuclear NF-κB p65. We postulated that such normal florae down-regulated inflammation by inhibiting NF-κB activation in gut epithelium.

Neish reported some nonvirulent Salmonella strains attenuated the synthesis of inflammatory effector molecules in model human epithelia elicited by proinflammatory stimuli by blockade of IκB degradation[16]. In order to investigate such an effect of the bacteria in gut, we selected and cultured two strains of bifidobacterium and lactobacillus under anaerobic conditions. The two strains successfully inhibited IL-8 secretion and NF-κB activation in intestinal epithelia in the experiment. At present, some probiotic compounds have been used in management of some diseases, such as maintenance therapy in IBD[17,18]. Although the mechanism of probiotic action has not been fully understood, the beneficial effects were consistent with an anti-inflammatory state conferred by probiotics[19,20]. More researches need to be done in order to understand the beneficial effect of probiotics on human beings.

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• CASE REPORT •

Dysentery caused by *Balantidium coli* in a patient with non-Hodgkin's lymphoma from Turkey

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Abstract

Balantidium coli is the only parasitic ciliate of man. It is a flattened oval organism covered with cilia, and a gullet at the anterior end. It is infrequently pathogenic for man, although epidemic buds in tropical zones have been described. The infection fundamentally affects the colon and causes variable clinic pictures, from asymptomatic to serious dysenteric forms. We present a case of parasitologically diagnosed as causes of diarrhea in a patient with non-Hodgkin's lymphoma from Turkey. In order to find out the causative etiologic agent of diarrhea, stool samples were examined by native, lugol and flotation methods and we detected moving trophozoites, which were approximately 60 μm long and 35 μm wide. These bodies were diagnosed as *Balantidium coli*. This case underlines that *Balantidium coli* should also be considered as a possible pathogen in immunocompromised patients with diarrhea.

Yazar S, Altuntas F, Sahin I, Atambay M. Dysentery caused by *Balantidium coli* in a patient with non-Hodgkin's lymphoma from Turkey. *World J Gastroenterol* 2004; 10(3):458-459
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INTRODUCTION

Balantidium coli (*B.coli*), the largest protozoon affecting humans, is a ciliate organism often associated with pigs. The greenish-yellow trophozoites may measure up to 120×150 μm and are capable of attacking the intestinal epithelium, creating ulcers and causing bloody diarrhea similar to that of amebic dysentery. It commonly infects primates, rats and pigs, and has a world-wide distribution. It is the only parasite of the family of *Balantidiidae* that, in rare instances, is pathogenic for humans. Balantidiasis is an infection of the large intestine by the ciliate protozoon, *B. coli*. In many infections (perhaps 80 percent) *B. coli* lives as a commensal in the lumen of the colon and causes no symptoms. A variety of gastrointestinal symptoms, including cramping, abdominal pain, nausea and foul breath, also occur. Encystment usually occurs in the intestinal lumen or stool (human or swine), and the large round cysts transmit the infection through contaminated food or water. Pigs act as carriers and are not often adversely affected by this organism[1,2].

Fortunately, balantidiasis is uncommon in temperate climates. It is found in association with pigs throughout the tropics, especially the Philippines[1]. Evidence indicates that some infected humans may become asymptomatic cyst carriers, whereas others clear the infection spontaneously. As with amebiasis, this condition probably runs the gamut between mild colitis and severe, potentially fatal dysentery. Treatment of adults and older children is usually accomplished with tetracycline, 500 mg four times daily for 20 days and metronidazole, 750 mg three times daily for five days[3].

Our study in this paper is the first reported case of balantidiasis in a patient with non-Hodgkin' s lymphoma from Turkey.

CASE REPORT

A 47 year-old female patient with non-Hodgkin' s lymphoma and complaining of diarrhea and abdominal pain was admitted to our hospital. In the patient' s history, there were watery, bad smelling, bloody diarrhea (ten times per day) and abdominal pain.

Physical examination revealed mild abdominal tenderness and increased sounds of intestine. In laboratory examination, blood routine tests were found as follows: Hb: 9.6 g/dl, Htc: 31.4%, white blood cells: 0.4×10^9/L (with 20% granulocytes and 80% lymphocytes), platelets: 56×10^9/L and sedimentation rate: 40 mm/h. Biochemical tests results were as follows: Fbg: 82 mg/dl, BUN: 40 mg/dl, creatinin: 0.7 mg/dl, total protein: 7.6 gr/dl, AST: 30 U/L, ALT: 32 U/L, ALP: 231 U/L, GGT: 43 U/L, LDH: 342 U/L, albumin: 4.1 gr/dl.

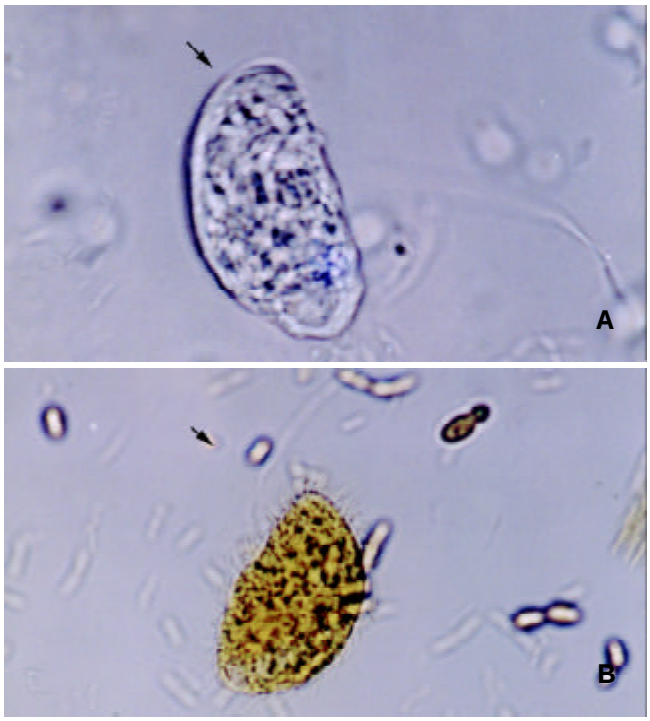

Figure 1 *Balantidium coli* trophozoites in native (A) and lugol (B) preparations (original magnification ×400).

We did not obtain any abnormalities in abdominal ultrasonography and direct X-ray. There was no growth of any pathogens in stool and blood culture.

In order to find the causative etiologic agent of diarrhea, stool samples were obtained from the patient and sent to the laboratory. The stool samples were examined parasitologically by native, lugol and flotation methods. Approximately 60 μm long and 35 μm broad moving trophozoites with cilia were seen. These bodies were diagnosed as *Balantidium coli* (Figure 1).

Consequently, we diagnosed this organism as *Balantidium coli*. The patient was treated with metronidazole 750 mg three times daily for five days. Following the treatment, the symptoms rapidly and completely resolved. Re-examination of a stool sample, however, did not reveal the presence of any organism.

DISCUSSION

Balantidium coli is a protozoon and the only one ciliate that is able to cause disease in man. The trophozoite or vegetative state has oval form covered by a great amount of cilia grouped in row. In its forcebody they are peristoma and citostoma that continue with citofaringe. The later end finishes in the anal pore or citopigio. The cysts are oval or spherical, they measure from 45 to 65 μm in length[2].

Although the distribution of *B.coli* is cosmopolitan, infection in humans takes place by the ingestion of cysts coming from lees of the parasite guests. Of this form, different epidemic buds like the one arising in the Truk Islands after a typhoon, have taken place. That typhoon caused an extensive contamination of superficial and underground water with lees of pigs, which provided the source of water for the population[4] and led to a severe prevalence in psychiatric hospitals[5,6]. Sporadic transmission was also produced by the water, contaminated vegetables crude and fecal-oral mechanism. People in contact with pigs were more likely to be infected, thus, it is said that the Aymara children of the Plateau of Bolivia had a greater prevalence of this infection, although usually they remained asymptomatic[7]. Our patient was personally questioned, she had not contacted with a pig. Probably, she might have got this parasite either via contaminated food or water. This case underlines that *B. coli* should also be considered as a possible pathogen in immunocompromised patients with diarrhea even if they have no contact with pigs.

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• CASE REPORT •

Unusual manifestations of gastric inflammatory fibroid polyp in a child

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Abstract

AIM: Inflammatory fibroid polyp (IFP) is a rare benign lesion that may occur throughout the digestive tract. IFP is more commonly found in the antrum of the stomach in particular. It mostly affects adults at the average age of 60 years. These polyps are able to cause abdominal pain, gastrointestinal bleeding, intestinal obstruction or intussusception. In this paper we report a case of gastric IFP with unusual presenting features.

METHODS: A child with gastric IFP was described and the literature was reviewed.

RESULTS: A 4-year-old girl presented with fever for 2 months, arthralgia of knees and ankles, iron deficiency anemia, and hypoalbuminemia. Her stool examination was positive for occult blood. The upper gastrointestinal study demonstrated a large lobulated mass at the upper part of gastric body. Partial gastrectomy *en bloc* with this 5cm×8 cm mass was subsequently performed. Pathological examination was consistent with IFP. Following the mass excision, her fever abruptly declined and disappeared together with anemia and arthralgia. She remained asymptomatic and the abdominal ultrasonography performed at the 24-month follow-up demonstrated no recurrence of the tumor.

CONCLUSION: The etiopathogenesis of IFP still remains unclear. The presence of IFP throughout the gastrointestinal tract and its variable clinical appearances make it difficult to diagnose. The inflammatory symptoms found in this patient support the hypothesis of inflammatory benign lesions of IFP.

Chongsrisawat V, Yimyeam P, Wisedopas N, Viravaidya D, Poovorawan Y. Unusual manifestations of gastric inflammatory fibroid polyp in a child. *World J Gastroenterol* 2004; 10(3): 460-462
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INTRODUCTION

Gastric neoplasms are exceedingly rare in children. Murphy *et al.* reviewed 1 403 pediatric gastric pathology reports and found only 3 benign gastric tumors[1]. Attard *et al.* reported hyperplastic-inflammatory polyp was the most common gastric polyps (42%) found in pediatric population[2].

Inflammatory fibroid polyp (IFP) is a solitary polypoid or sessile lesion with an inflammatory basis. It is a rare benign lesion that may occur throughout the digestive tract, but is most often seen in the stomach (approximately 80%)[3]. IFP in the stomach is usually located in the antrum or prepyloric region[4,5]. In large retrospective studies of gastric polyps, 3.1-4.5% were found to be IFP[6,7]. It is slightly more common in women (female:male ratio 1.6:1)[5]. It is found in all age groups, although not often in children, and its maximal incidence is in the sixth decade[7]. The symptomatology is determined by its site. In the stomach, it causes pyloric obstruction, and often in the small bowel, intussusception which is the most common presentation in children.

We report a case of gastric IFP who presented with prolonged fever, arthralgia, hypoalbuminemia, and iron deficiency anemia. Surgical excision led to a complete resolution of those symptoms.

CASE REPORT

A 4-year-old girl presented with high fever for 2 months, arthralgia of knees and ankles, and anemia that required multiple packed red cell transfusions. The lowest hemoglobin was 3.9 g/dL. A provisional diagnosis of juvenile rheumatoid arthritis was made. She was treated with aspirin and prednisolone, but had no improvement. Later she was referred to our hospital in June 2001.

On her admission, the patient' s weight was 15 kg (25^{th} percentile for gender and age). Physical examination revealed pale conjunctivae, pitting edema of both legs, the rest was unremarkable. Laboratory tests after multiple blood transfusions showed a white blood cell count of 41 400/mm^3, 76% PMNs, 16% lymphocytes, 6% monocytes, and 2% atypical lymphocytes; hemoglobin was 11.4 g/dL (MCV 76 fL, MCH 21.1 pg, MCHC 27.9 g/dL, RDW 21.2%), and platelet count was 684 000/mm^3. Stool occult blood was positive. Serum albumin and globulin were 2.1 g/dL and 3.2 g/dL, respectively. Anti DNA, ANA, ANCA, anti Smith, rheumatoid factor, VDRL and LE cell were negative. β_1C was 176.9 mg/dL. ESR was 82 mm/h. Culture of blood, urine, stool, and bone marrow aspirate were negative. Upper GI series demonstrated a large lobulated mass at the upper part of gastric body (Figure 1).

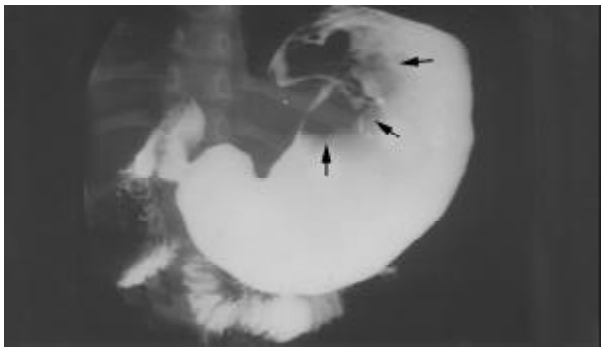

Figure 1 A large lobulated mass at the upper part of gastric body (arrow head) shown by radiography.

Table 1 Literature review of inflammatory fibroid polyp in children (since 1960 in English language)

Year	Reference	Age (y.)	Sex	Location	Clinical manifestations
1966	Samter[8]	4	Male	Colon	Abdominal pain, vomiting, concomitant ruptured colonic diverticulum
1966	Samter[8]	8	Female	Jejunum	Vomiting, intermittent diarrhea, anemia
1972	Persoff[9]	3	Male	Ileum	Abdominal pain, vomiting, diarrhea
1984	Pollice[10]	8	Male	Rectum	Lower GI bleeding, anemia
1987	Schroeder[11]	5	Female	Stomach	Abdominal pain, weakness, vomiting, anemia
Our study	Chongsrisawat	4	Female	Stomach	Prolonged fever, arthralgia, anemia, hypoalbuminemia

At laparotomy, a 5 cm×8 cm mass was found at the body of the stomach. Partial gastrectomy *en bloc* with the mass was performed. Light microscopic examination of the gastric mass showed foci of ulcerated mucosa, focal elongated distorted branching and dilated hyperplastic foveolar and glands, covering a mass consisting of proliferative fibroblasts and blood vessels admixed with mixed inflammatory cell infiltrates in the stroma (Figure 2). The histological finding was consistent with IFP.

The patient developed postoperative convulsion due to steroid-induced hypertension and encephalopathy. She was treated with phenytoin and nifedipine which were later discontinued after 2 and 4 months, respectively. Fever, arthralgia, hypoalbuminemia, and anemia resolved after removal of the polyp. She was discharged 12 days after the surgery. The abdominal ultrasonography performed at the 24-month follow-up demonstrated no recurrence of the tumor and she remained asymptomatic.

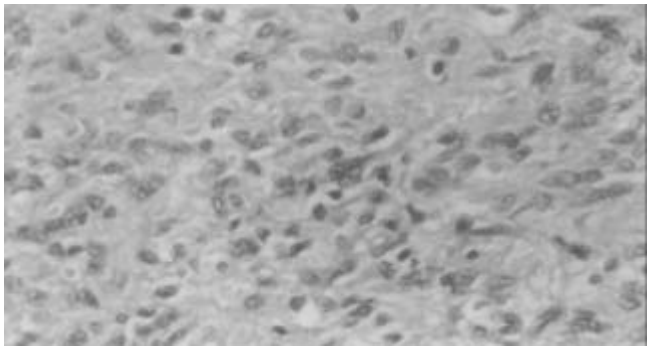

Figure 2 Packed spindle-shaped cells, proliferated vessels and inflammatory cells, mostly eosinophils observed in tumor (H&E, 10×40).

DISCUSSION

Current pediatric literature regarding IFP is limited to case reports and small case series as shown in Table 1. Clinical manifestations of IFP are variable, depending on the location and size of the lesion. Most are small and asymptomatic. Nevertheless, it is able to cause anemia due to gastrointestinal bleeding, abdominal pain, vomiting, weight loss, intestinal obstruction or intussusception. Physical examination is usually not conclusive. Imagings such as upper GI series, ultrasonography or computed tomography can help diagnose the mass, but the final diagnosis is generally based on endoscopy and histological examination.

Histologically IFP has been found to be characterized by a submucosal lesion with a mixture of proliferation of fibroblasts and small blood vessels, accompanying a marked eosinophilic infiltration[12]. Since the distinctive structures for diagnosis are located within the submucosa and at the base of the mucosa, the diagnosis may not be possible in most of these polyps by endoscopic biopsy specimen. In the past, IFP was reported under several terms, such as eosinophilic granuloma, submucosal granuloma with eosinophilic infiltration, hemangioendothelioma, hemangiopericytoma, and polypoid fibroma[11,13]. The etiology and the pathogenesis of IFP remain unclear. It has been hypothesized that several factors could damage the gastrointestinal mucosa and expose the stroma to several irritants (chemical, mechanical and biological), and stimulate the formation of polyps among certain people[14]. A polyp of this category is a specific response of gastrointestinal stromal tissue of unknown etiology. Electron microscopic study revealed that IFP represented a reactive lesion of myofibroblastic nature[15]. Thus, it has been now generally accepted that IFP is not a neoplasia, but a reactive process, either to an allergy or a foreign body and has no malignant potential[4-6].

An infective etiology has never been reported in IFP, although several reported cases showed its association with *Helicobacter pylori* infection[3,14]. In one of these reports, the patient also had autoimmune diseases (sarcoidosis, rheumatoid arthritis, and ankylosing spondylitis)[3]. This finding supports the possibility of an immunological reaction as a contributing factor. It is not exactly known that *H pylori* started an immunological reaction which caused the polyp, or it only complicated this finding. Surgery is the main therapy of IFP and the recurrence has not been observed after polyp removal. Small polyps up to 2 cm in diameter can be excised endoscopically.

Our case is unusual in several aspects, such as the patient' s young age at the presentation, the location of the mass at the corpus of the stomach, and the autoimmune-like manifestations. We hypothesized that IFP might release cytokines that cause fever and other autoimmune-like symptoms in this patient.

CONCLUSION

Not only is IFP rare in children, but its presence throughout the gastrointestinal tract and its variable clinical appearance make it difficult to diagnose. Patients with IFP may present with inflammatory symptoms and disappearance of the symptoms after polyp removal found in this patient supports the hypothesis of inflammatory benign lesions of IFP.

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