Editorials

Gastrin as an autocrine growth factor in colorectal carcinoma: implications for therapy

Graham S. Baldwin and Arthur Shulkes

Subject headings Autocrine loop; colorectal neoplasms; gastrin; gastrin receptor; progastrin

There is now considerable experimental support for the hypothesis that progastrinderived peptides stimulate proliferation of the normal colonic mucosa[1], and act as autocrine growth factors in colorectal carcinoma (CRC). In a previous review[2] we summarized the evidence for the presence of progastrin-derived peptides and their receptors in CRC, and presented a model in which amidated and non-amidated progastrin-derived peptides stimulate proliferation of CRC cells via distinct receptor classes. In this editorial we will consider the various strategies available to interfere with the individual components of the autocrine loop, and the potential of the strategies to yield novel diagnostic and therapeutic agents.

AUTOCRINE LOOPS

In the autocrine model (Figure 1) a cell synthesises its own growth factor, which is released into the surrounding medium. Binding of the growth factor to cell surface receptors then results in the transmission of a mitogenic signal to the cell nucleus, with a consequent increase in cell proliferation. In principle, autocrine loops could be disrupted in at least 3 ways:

1. By reduction of growth factor mRNA with introduction of antisense RNA or oligonucleotides,

2. By reduction of the concentration of extracellular growth factor by treatment with specific antibodies, or

3. By blockade of growth factor receptors with selective antagonists.

In addition the presence of elevated concentrations of tumour-derived growth factors in the sera of patients with CRC may offer a sensitive method of tumour detection, via the development of anti-growth factor antibodies suitable for radioimmunoassay.

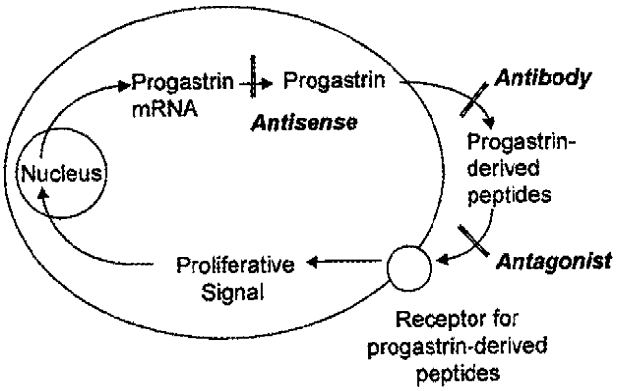

Figure 1 An autocrine growth loop involving progastrin-derived peptides.

ANTISENSE mRNA EXPRESSION

Antisense experiments have provided clear evidence for the involvement of progastrinderived peptides in an autocrine loop in some cell lines of colonic origin. Expression of antisense gastrin mRNA reduced *in vitro* growth of the CRC cell lines Colo 320 and HCT 116[3]. and of the conditionally immortalized mouse colon cell line YAMC[4]. The ability of HCT 116 cells to grow as tumours in nude mice was also reduced by antisense gastrin mRNA expression[3]. In control experiments *in vitro* and *in vivo* growth of the CRC cell line Colo 205A, which expressed negligible amounts of gastrin mRNA prior to transfection, was unaffected by expression of antisense gastrin mRNA[3]. However the inherent difficulty of selectively targeting antisense constructs to tumour cells may delay development of related clinical therapies.

ANTIBODIES

Diagnosis

The question of whether or not CRCs produce progastrin-derived peptides has been controversial,

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Received 1998-10-28

at least partly because of the large number of potential products of the gastrin gene[2]. Progastrin is processed to amidated gastrin via a number of intermediates which include glycine-extended gastrins. Some early reports were confined to measurement of amidated forms of gastrin only, and the variable extent of postranslational processing of progastrin in peptide-producing tumours may explain some of the negative findings reported in the literature. Progastrin or progastrin-derived peptides are now detected in 80%-100% of CRCs[2]. The concentrations of progastrin-derived peptides in the serum of patients with CRC are also elevated between 2.3-fold (*H. pylori* negative) and 5.2-fold (*H. pylori* positive)[5]. The availability of a panel of antibodies recognizing different regions of intact progastrin, and of antibodies selective for individual progastrin-derived peptides, may permit the early diagnosis of CRC by radioimmunoassay of serum samples. In this context a large prospective study has recently indicated that hypergastrinaemia was associated with a 3.9-fold increase in the risk of later development of CRC[6].

Therapy

Antibodies against progastrin-derived peptides may also be useful for treatment of CRC. The proliferation of some, but not all, CRC cell lines was inhibited by antibodies recognizing the C-terminal amidated tetrapeptide of gastrin[2]. On the other hand, proliferation of the mouse colon cell line YAMC was inhibited by antibodies recognizing glycine-extended, but not amidated, gastrins[4]. A promising approach to future therapy has been provided by the observation that preimmunization of rats with Gastrimmune (a conjugate of amino acids 1-9 of gastrin17 and diphtheria toxoid which recognises both gastrin17 and gastrin17-gly) reduced the *in vivo* growth of the rat CRC cell line DHDK12, either alone or in conjuction with 5-fluorouracil and leucovorin[7].

ANTAGONISTS

Gastrin/CCK receptor antagonists

At least four receptors exist for the gastrin/CCK family of peptides2. The CCK-A and gastrin/CCK-B receptors are specific for amidated peptides, while the glycine-extended gastrin receptor is selective for non-amidated forms of gastrin. The low affinity gastrin/CCK-C receptor binds amidated and non-amidated forms of gastrin with equal affinity. While the nonselective antagonists proglumide and benzotript inhibit the binding to gastrin/CCK-A, B and C receptors, antagonists selective for either-A or -B receptors have also been developed[2].

The non-selective antagonists proglumide and benzotript inhibit proliferation of many gastrointestinal carcinoma cell lines both *in vitro* and *in vivo*[8]. Comparison of the inhibitory potencies of proglumide, benzotript and other selective gastrin/CCK receptor antagonists with receptor affinities suggests that the gastrin/CCK-C receptor is the probable target[9]. However a clinical trial of proglumide in patients with gastric carcinoma did not reveal any benefits, perhaps because the concentrations achieved were not sufficient to saturate gastrin/CCK receptors. Gastrin/CCK-B receptor antagonists have also been shown to inhibit the growth of some CRC cell lines *in vitro*, and of primary human CRCs *in vitro* and *in vivo*, but have not yet been subjected to clinical trials. However the observation that most CRCs do not express gastrin/CCK-B receptors indicates that it will be unlikely that gastrin/CCK-B receptorselective antagonists will be a general treatment for CRC[2].

Non-steroidal anti-inflammatory drugs

Epidemiological studies have revealed that non-steroidal anti-inflammatory drugs (NSAIDs), and in particular aspirin, reduce by approximately 50% the risk of CRC and other cancers of the gastrointestinal tract[10]. The NSAID sulindac also reduces the size and number of colorectal polyps in patients with familial adenomatous polyposis, and inhibits the development of chemically-induced CRC in rodents. Although selective antagonists have indicated that the inducible isozyme cyclooxygenase-2 is one of the targets for the inhibitory effects of NSAIDs on CRC growth *in vivo*, several lines of evidence suggest that other targets may contribute to the anti-proliferative effects *in vitro*[10].

The gastrin/CCK-C receptor may be one such alternative target. All of a panel of 17 NSAIDs tested inhibited the binding of gastrin to the gastrin/CCK-C receptor with affinities which correlated well with their potencies as inhibitors of the proliferation of CRC cell lines[11]. The most potent antagonist of gastrin binding to date is sulindac sulphide, which has an IC_{50} value of 40μM, and more potent antagonists of the gastrin/CCK-C receptor may well be of use in the treatment of CRC.

CONCLUSIONS

This editorial has summarized several promising avenues for future research into the effects of progastrin-derived peptides as autocrine growth factors in CRC. In particular the development of

antibodies against progastrin-derived peptides, and of antagonists selective for progastrin-derived peptide receptors, may provide new opportunities for diagnosis and therapy of CRC.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):464-466

Immune response against HBsAg vaccine *

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Subject headings rHBsAg; hepatitis B; vaccines; vaccination

Hepatitis B virus (HBV) is a hepadnavirus which can cause systemic infection and hepatocellular damage in humans. Infection is acquired through contact with the blood of a person carrying HBV. The passive administration of anti-HBV surface antigen (HBsAg) antibodies protects against a subsequent infection and vaccination with HBsAg has proved an effective means of protection against HBV infection.

HBsAg purified from the plasma of HBsAg-carriers has been used for many years as an effective vaccine but its major limitation is the availability of the raw material and the cost. Since the late 1980s, genetic engineering has allowed the development of yeast-derived recombinant, very safe and efficient DNA vaccines[1] and compulsory vaccination was introduced in many countries to progressively eradicate HBV infection. Recombinant vaccines contain only the sequence 1-226 of HBsAg (rHBsAg), representing the small nonglycosylated HBs protein (S-domain), with the exclusion of the preS1 and preS2 domains (Figure 1).

A serum level of at least 10 mIU/ml of anti-HBs antibodies reached after vaccination has been proposed to be the lowest limit for protection[2]. Several studies were conducted to examine the duration of protection after immunization with recombinant vaccines, but these were related to seroconversion in selected clusters of vaccine recipients, introducing parameters (age, gender, smoking, obesity, etc.) which are known to influence the immune response[3,4]. Recently, we and others proposed a mathematical model to calculate the time after the vaccination with rHBsAg for the loss of protection due to an antibody fall to below 10 mIU/ml[5,6]. This model, which will beavailable on the web-6, showed that the decay in the level of specific immunoglobulins reached after last booster dose is well described by the log time-log titre linear function. In practice, anti-HBs level falls quickly after the peak and then slowly, and the time to reach very low levels depends on the serum level reached at the end of the vaccination protocol.

The analysis of specificity of raised circulating antibodies after inoculation with rHBsAg showed a binding region for one B-cell epitope represented by a peptide located within the HBsAg sequence 110-168 (Table 1). The same specificity has been described after immunization with plasma-derived vaccines or natural infection. This epitope represents the 《a》 determinant of HBsAg, that may be considered the conserved sequence among different HBV subtypes (adr, adw, ayr, ayw) and is able to stimulate cross-protection after immunisation[7]. This dominant B-cell epitope is conformational, discontinuous, characterized by disulphide bonds and corresponds to a hydrophilic, external region of HBsAg. Although antibodies directed against this region protect against HBV infection, an escape mutant of HBV has been isolated from an infant vaccinated with rHBsAg and later became positive for markers of viral replication. The mutant presents an amino acid substitution of glycine to arginine at position 147[8]. This point mutation is stable, allows the attachment of the virus to hepatocytes and represents a new specificity not cross-reacting with normal virus subtypes. The mutation of one amino acid can be responsible for different conformations of 《a》 determinant, with varying abilities to elicit and bind anti-HBs antibodies.

Although the immunogenicity and protective effect of rHBsAg are comparable with that of HBV natural infection, differences have been described in IgG subclass distribution after infection or vaccination. The serological response to HBsAg has been well characterized in patients with acute or chronic hepatitis B, who presented circulating immunocomplexes containing mostly IgG1 and IgG4 antibodies, or in convalescent sera where specific antibodies were predominantly IgG1 and IgG3. The analysis of IgG subclass distribution induced by vaccination with rHBsAg shows that the IgG2 subclass is mainly synthesized with IgG1. The presence of IgG2 with IgG1 subclass following

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***Supported in part by Grants from the Italian Ministry of Health, University of Bologna (ex60%) and Ricerca Corrente, Istituti Ortopedici Rizzoli, Bologna, Italy.**

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Received 1998-11-16

vaccination against HBV was also described using plasma-derived vaccines, where HBsAg is glycosylated. Although the synthesis of IgG2 subclass antibodies is generally associated with polysaccharide antigens, the absence of glycosylated residues in the rHBsAg cannot explain the switch to IgG2 production. A hypothesis to explain a comparable immunogenicity from both vaccines is that the network of cytokines secreted by T lymphocytes recognising natural or recombinant HBsAg, could induce the B lymphocyte switch to this IgG subclass synthesis[9].

Table 1 B and T cells epitopes of HBsAg

Sequence	Determinants	Cell subsets	HLA restriction
110-168[6]	Conformational《a》determinant	B	Not HLA restricted
21-28[11]	Minimal epitope	CD4 T	Class II
124-147[11]	Immunodominant zone	CD4 T	Class II
165-172[11]	Minimal epitope	CD4 T	Class II
215-223[11]	Minimal epitope	CD4 T	Class II
80-98[11]	Containing predicted epitope	CD4 T	Class II
171-179[16]	Minimal epitope	CD8 T	Class I

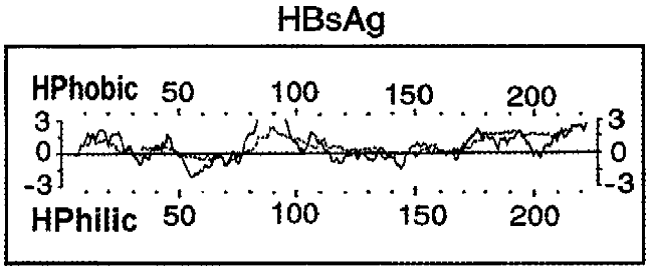

Figure 1 Structure of the HBsAg molecule. The hydropathy of the sequence 1-226 is predicted according to Kyte and Doolittle 1982, J Mol Biol (——) and Engelman *et al*. 1986, Annu Rev Biophys Biophys Chem (···).

Anti-HBS production is the consequence of T cell-mediated immunity stimulated by the vaccination, which also plays a fundamental role in preventing HBV infection. The use of genetic engineering to synthesize recombinant HBV envelope protein increased investigation into the immunising activity of HBsAg. Some early studies *in vitro* disclosed that CD4+T cells help HBsAg specific B cell for the synthesis of anti-HBs antibodies in subjects vaccinated with rHBsAg[10,11].

The isolation of T cell clones specific for HBsAg from subjects vaccinated with recombinant vaccine provided an important tool for investigating the immunizing activity of this protein. It has been observed that the response of antigen-specific T lymphocytes to HBsAg depends on the sequence of the antigen molecule. In an attempt to identify the sequences recognized by HBsAg-primed T cells from vaccine recipients, synthetic peptides representing short sequences of HBsAg protein have been used *in vitro* to investigate specific cellular proliferation. Recently, immunodominant HBsAg epitopes have been confirmed or identified analysing the proliferation of CD4+T cell clones (helper/inducer) by peptides spanning the whole HBsAg protein[12]. The clones belong to the Th_0/Th_2 subset, expressing and secreting *in vitro* interleukin 4, 5 and interferon-γ. Three minimal T-cell epitopes have been described: sequences 21 - 28, 165-172 and 215-223. There is an evidence that T-cell epitopes are also present within the 124-147 sequence, corresponding to that zone on HBsAg containing the 《a》 determinant. The difficulty in identifying a minimal epitope within this sequence could be due to the low level of immunogenicity of this HBsAg region. We cannot exclude the presence of other important epitopes with in the most hydrophobic region of HBsAg, that is very difficult to study because of this characteristic, which prevents the solubility of peptides in culture media (Table 1). These findings are in agreement with the Berzofsky algorithm[13], which predicts for different protein zones the probability of representing Thepitopes on the basis of the sequential and conformational structure. The pathway of exogenous antigen processing needs the cooperation of antigen presenting cells (APC) and the recognition of short aminoacid sequences (epitopes) by Th cells in association with HLA class II determinants. Recently it has been shown that: ① DR molecules are mostly involved in the presentation of immunodominant HBsAg peptides, but at least DP determinants can participate in antigen presentation; and ② an epitope can be efficiently presented by different MHC loci, suggesting that a short sequence of HBsAg can be immunogenically dominant, because of its specificity for Th cells from subjects presenting different HLA class II alleles[12]. On the other hand, studies on the contribution of HLA class II determinants to the regulation of antibody production suggested that MHC loci are involved in regulation of the immunological response after rHBsAg immunization.[14].

In vitro studies on CD8+T cells responsible for cytotoxic activity are hampered by the difficulty of utilising target cells expressing appropriate HLA class II determinants for the association with the antigen. Although the cytotoxic activity of CD8+T cells stimulated by HBsAg is not well known, one minimal essential epitope on HBsAg has been

described in sequence 172-180[15]corresponding to a peptide candidate for the binding to HLA-A2 determinant and this sequence has been described to activate T CD8+ lymphocytes isolated from subjects sharing this HLA class II determinant. It is also noteworthy that this antigen can evoke cytotoxic activity by subsets of CD4+T cells expressing the CD56 phenotype[16]. We do not exclude that different short sequences of S protein could induce an immunological response involving both CD4+ and CD8+T lymphocytes. Double pathway for the antigen processing, utilising endosomic and cytosomic cellular districts, has been described for the pre S2 protein sequence 120-134 of HBV, which can be presented by HLA class I and class II determinants, leading to activation of CD4+ (helper/inducer) and CD8+ (cytotoxic) T cells[17]. Identification of dominant epitopes on HBsAg specific B, CD4+ and CD8+T cells may clarify the effective role of rHBsAg in inducing a protective cellular and humoral response against HBV infection. The major histocompatibility complex (MHC) controls the immune response to protein antigen and allelic variants of MHC can influence the lack of response to the immunisation in a small percentage of vaccine recipients (5%-10%).

More recently some authors have analyzed the possibility of utilising HBsAg-encoding plasmid DNA as vaccine. Good findings have been obtained in vivo following i.m. injection of the plasmid into mice[18]. This route of administration induced muscular cells to synthesize endogenous HBsAg followed by the association of peptides with HLA class I determinants and the secretion of the whole molecule. In the future, DNA vaccine could be considered an alternative method to obtain an active immunization to several pathogens if this approach proves to be as effective and safe as recombinant protein.

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The role of adhesion molecules in gastric ulcer healing

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Subject headings stomach ulcer; adhesion molecules; vascular cell surface adhesion molecul-1; platelet endothelial cell addesion molecule

Gastric ulcer is a deep necrotic lesion involving the entire mucosal depth and the muscularis mucosae. Ulcer healing is an active and complicated process of filling the mucosal defect with proliferating and migrating epithelial cells and connective tissue components, so as to reconstruct the mucosal architecture. In this process, the concerted interaction of a variety of tissues and cellular systems are reguired, including those of soluble mediators, formed blood elements, extracellular matrix (ECM), and parenchymal cells. In fact, healing of an ulcer follows a specific time sequence and can be temporally categorized into three processes which occur in a sequential order: (I) inflammation; (II) tissue formation; and (III) tissue remolding. This editorial will mainly discuss the involvement of adhesion molecules in the phase of tissue formation, a fundamentally important process in ulcer healing

The critical steps of tissue formation during the repair of the gut mucosal injuries include the reconstitution of the epithelial mucosal barrier and the development of new microvasculature by angiogenesis. The healing of the gut is initiated by restitution. Enterocytes at the edge of the wound lose their differentiated characteristics and migrate across the region of denuded basement membrane in mucosa, while stimulation of angiogenesis during initial wound healing facilitates the reconstruction of original appearing mucosa and submucosa. These processes could be initiated by a variety of soluble chemotactic cytokines and growth factors secreted from damaged cells.

Epithelial restitution is a fundamental protective mechanism that allows the gastrointestinal mucosa to reestablish functional and structural integrity following superficial injury. The initial step and progression of epithelial cell migration depends on the adherence between epithelial cells and establishment of stable cell-substratum adhesion to generate friction and forward movement. The transformation from an attached to a motile cell requires disruption of cell junctional proteins such as E-cadherin/ catenin complex and the modulation of expression, affinity and binding specificity of ECM adhesion receptors (e.g., integrins). Integrins have been implicated in the regulation of cell migration in a variety of systems[1]. Knock-out of β1 integrin gene in embryonic stem cells has been shown to inhibit cell migration and adhesion[2]. The up-regulation of the functional activity of integrins α2β1 and α3β1, which are receptors for laminin and collagen, is required for cell attachment and migration. Epithelial cadherin is the prime mediator of cell-cell adhesion in epithelial cells. Perturbation of E-cadherin/ catenin mediated adhesion is as sociated with epithelial migration and restitution following ulceration of the gastrointestinal tract[3]. Epidermal growth factor has been shown to induce rapid tyrosine phosphorylation of β-catenin and γ-catenin. This is associated with scattering and dispersion of epithelial cells and disruption of E-cadherin from the cell-cell junctions[4].

Angiogenesis is another crucial factor for ulcer healing and tissue regeneration[5], which is a process of new blood vessel formation from pre-existed vessels. The importance of angiogenesis in gastroduodenal u lcer healing has been extensively studied. For instance, stimulation of angiogenesis in granulation tissues has been shown to dramatically accelerate the healing of experimental duodenal ulcer in rats[6]. Furthermore, chronic indomethacin administration inhibits angiogenesis in granulation tissues and delays healing of experimental gastric ulcers in animals[7]. Blood vessels are especially important during tissue injury. When there is inflammation, blood delivers nutrients, growth factors, and immunocytes to the site of injury, whereas waste products are removed from there. In addition, regulation of blood flow in the gastrointestinal mucosa is important for the maintenance of the integrity of gastric mucosa and protection against further mucosal injury. The formation of new vessels in ulcer healing is a dynamic process that is controlled by many diverse, sometimes complex factors acting together in a local environment. Again, a wide variety of growth factors are involved in the regulation of adhesion

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Received 1998-10-19

molecule expression in a concerted manner during the process of vessel assembly. The principal cell type involved in the process of angiogenesis is the microvascular endothelial cell.

Angiogenesis begins with proteolysis of the basement membrane. Proteolysis is necessary to induce microvascular endothelial cell invasion and tube formation. Activation of both endothelial cells and lymphocytes or monocytes is required for their secretion of proteases. Their activation, however, is dependent on adhesion molecules interaction. During the course of inflammation, local endothelial cells are exposed to various cytokines that induce a series of endothelial surface adhesion molecules. One of these inducible molecules, vascular cell surface adhesion molecule-1 (VCAM-1), a member of the immunoglobulin (Ig) supergene family, is the counter-receptor for very late antigen-4 (VLA-4), a surface protein present on lymphocytes and monocytes. VCAM-1/VLA-4 interaction induces the increased expression of a series of proteases required for proteolysis, and therefore endothelial cells could get a hold on the basement membrane, where they proliferate and extend.

Following proteolysis, tube formation occurs. A series of adhesion molecules come to play in tube formation. Early events of tube formation are mediated by platelet endothelial cell adhesion molecule (PECAM-1/PECAM-1) interactions. As a result of the cell to cell contact, a tube-like structure is formed. Antibodies directed against PECAM-1 can inhibit tube formation *in vitro*[8]. Integrin αvβ3 is another adhesion molecule found only on the tips of the endothelial cells in sprouting vessels. Its immunoreactivity is absent in mature and quiescent vessels. Both β1 and β3 integrins are involved in the attachment between cells and their substrates. But the presence of the latter adhesion molecule was also thought to induce gelatinase expression on the surface of the endothelial cells so as to enhance migration and proliferation[9]. When the tube-like structure is stabilized with β1 and β3 integrins, tight junction formation occurs, which correlates with the junction-associated molecules assembly and organization[10].

Epithelial restitution, as well as angiogenesis, two of the fundamental components of the ulcer healing process, are characterized by complex alterations in adhesion between cells and the ECM. Growth and motility factors involved in mucosal repair of the gastrointestinal tract seem to modulate these interactions in a coordinated fashion in order to reestablish functional and structural integrity of the mucosa. The mechanisms that regulate the production of these adhesion molecules await further exploration and clarification, as do the differences between the consequences of different types of mucosal injury. It is clear, however, that the modulation of the cell migration, and angiogenesis by adhesion molecules may be the fruitful targets for future pharmacological intervention in gastrointestinal wound healing.

Acknowledgement: This review is supported in part by the RGC grant of the Hong Kong Research Grant Council, awarded to C.H. Cho.

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Phytochemical malabsorption: clinical significance

Mark L Wahlqvist and Naiyana Wattanapenpaiboon

Subject headings phytochemicals;vitamins;minerals; gastrointestinal malabsorption

CLINICAL SIGNIFICANCE OF PHYTOCHEMICALS

Until recent years, nutritionists have focused primarily on macronutrients and micronutrients in foods. Appreciation is now increasing that many food components, of plant origin ('phytochemicals') in particular, have the potential to affect human biology. Phytochemicals by definition are important components of food that may not be essential in the classical sense, and may not even be required to sustain life as vitamins or minerals do, but are likely to contribute to optimal health.

A problem in considering the place of phytochemicals in human health is that they are numerous, alongside a few known essential nutrients. Therefore, their net interactive effect ultimately requires a study of food itself and food patterns, so that food component intake may need to be subject to sophisticated mathematical modelling. However, the advant of advanced informatics may help resolve this dilemma.

Examples of phytochemicals, along with some of their food sources are listed according to their chemical structure in Table 1. The possible roles of these phytochemicals in the treatment of health conditions are rapidly unfolding[1], and the use of an index of preferred phytochemical intake has been suggested[2]. Yet limited information is available on the bioavailability of most of these compounds. With complex factors influencing the absorption and transport of phytochemicals, it is not easy to predict their bioavailability, let alone consider the implications of gastrointestinal malabsorption.

BIOLOGICAL OCCURRENCE AND RELEVANCE OF PHYTOCHEMICALS

The presence and the physiological concentration of phytochemicals in biological tissues or fluids, especially blood and urine, create the opportunity for biomarkers of the consumption of phytochemical-containing foods[4-6] (Tabe 2). Equally, measurements of phytochemicals reflect bioavailability, including absorption.

Table 1 Selected phytochemicals and their possible roles in health[3]

Phytochemicals	Some important food sources	Possible roles in health
Carctenoids	Orange pigmented and green leafy vegetables, e.g. carrots, tomatoes, spinach	Antioxidants Antimutagen Anticarcinogen Immuno-enhancement
Flavonoids, isoflavonoids and saponins	Green and yellow leafy vegetables, e.g. parsley, celery, soy bean and soy products	Antioxidants Anticarcinogen Oestrogenic Immuno-modulating
Polyphenols	Cranberry, raspberries, blackberries	Antioxidants Antibacterial
	Rosemary, oregano, thyme	Reduce urinary tract infection
Calechins	Green tea	Antimutagen Anticarcinogen Anticariogen
Allyl thiosulfinates	Garlic, onions, leeks	Anticarcinogen Antibacterial Cholesterol lowering
Isolhiocyanates and indoles	Cruciferous vegetables, e.g. broccoli, cabbage	Antimutagen
Phytosterols, e.g. β-sitosterol	Pumpkin seeds	Reduce symptoms of prostate enlargement

This list is not exhaustive for phytochemicals.

Table 2 Occurrence of phytochemicals in human blood and tissues

Phytochemicals	Where can we find them in the body
Carotenoids	Serum (five major carotenoids)
Lutein / zeaxanthin	Skin
β-cryptoxanthin	Adipose tissues
Lycopene	Lens and macula (lutein / zeaxanthin)
α-carotene	Various tissues like prostate (lycopene)
β-carotene	
Flavonoids	Serum
Quercetin, kaempferol	Urine
Isoflavcnes	Serum
Geristein, daidzein	Urine
Catechins eplgallo-catechin gallate	Serum
Allyl thiosulfinates	Blood, serum, red blood cells
organosulfides	Adipose tissue
vinyl dithiins	Liver
	Kidney
	Breath
Tocotrienols	Sikin

For the moment, the presence of phytochemicals in tissues, is presumptive evidence of functional

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Received 1998-10-20

significance, especially where there is a plausible mechonism of action (e.g. carotenoids as antioxidants for serum lipoproteins). Observations like this generate a number of exciting hypotheses for further research.

CLINICAL SIGNIFICANCE OF PHYTOCHEMICAL MALABSORPTION

Given the putative health benefits of phytochemicals, gastrointestinal malabsorption may well contribute to a loss of their protective effects. This could result in a number of clinical disorders which could be referred to as 'phytochemical deficiency disorders'[1]. Candidate disorders are:

a. The menopause as a 'phytoestrogen deficiency disorder'[7]

b. Cardiovascular disease because of the role of certain phytochemicals as antioxidants, others as are regulators of endothelial function and others as modulators of myocardial function

c. Colorectal cancer in relation to a range of phytochemical intakes from various fruits and vegetables, and whole grain cereals[8]

d. Prostatic disease in relation to lycopene and isoflavones[9,10]

e. Maculopathy on account of the contribution to macular function of lutein and zeaxanthin[11]

Two cases of short bowel syndrome where carotenoids were undetectable in serum illustrate the potential for these orders (Case reports 1 and 2). In each case, it was possible to increase serum carotenoid concentrations by vegetable juice supplements.

Case report 1 Mrs BW (b. 1956)

Vaginal cancer (1990) treated with radiotherapy

Rectovaginal fistula - colostomy (1992)

Short bowel syndrome (1995)

Vegetable juice / soup		No	Yes
Serum concentration (nmol / L)	Reference range	Jan 96	May 97
Lutein / zeaxanthin	80-850	102	202
β-cryptoxanthin	175-1350	Not detectable	12
Lycopene	69-650	Not detectable	33
α-carotene	15-300	Not detectable	Not detectable
β-carotene	45-900	Not detectable	93

Case report 2. Mrs CM (b. 1956)

Severe road traffic accident (1976)→ruptured bowel, bowel resections

Short bowel syndrome

V-8ceTM (glass / day)		0	1×2	1×1
Serum concentration (nmol / L)	Reference range	Nov 95	May 96	Nov 96
Lutein / zeaxanthin	80-850	Not detectable	243	17
β-crytoxanthin	175-1350	Not detectable	18	Not detectable
Lycopene	69-650	Not detectable	32	11
α-carotene	15-300	Not detectable	Not detectable	Not detectable
β-carotene	45-900	Not detectable	10	8

CONCLUSIONS

Witn arnergent evidence for physiological roles of phytochemicals and for their potential for disease protection, the use of foods, which are good phytochemical sources, to prevent and manage disease will be encouraged. The malabsorption of phytochemicals is likely to be one of many pathways to so-called "phytochemical deficiency disorders".

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Clinical research advances in primary liver cancer

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Subject headings liver neoplasms/surgery; hepatectomy; liver neoplasms/therapy

Primary liver cancer (PLC) is one of the most common cancers in China. According to the statistics of our country, primary liver cancer claims 20-40 lives per100 000 people annually, with 19.98 per100 000 in cities and 23.59 per 100 000 in rural areas, ranking as the second and the first leading cause of cancer death respectively. Of all the newly enrolled cases in the world each year, 45% are found in the mainland of China. In the southeast areas of high incidence, the situation is even worse that the tumor tends to occur in a younger age group.

In China, the research in the diagnosis and treatment of primary liver cancer has undergone four stages: ① in the 1950s, the anatomical study of the liver lay a solid foundation for liver resection. ② In the 1960s and 1970s, as the detection of AFP and other liver cancer markers were widely used, the ability of early diagnosis was greatly improved with a better therapeutic effect. ③ In the 1980s, the introduction of some new techniques, such as CT, MRI, DSA, Doppler ultrasonography, etc., some new methods, such as hepatic artery chemoembolization (TACE), percutaneous intra-tumor ethanol injection (PEI), hepatic artery ligation (HAL) plus catheterized chemotherapy and target therapy, and some new concepts, such as curative local resection, reoperation of the recurrent liver cancer, two-stage resection, the combined surgical management of liver cancer complicated with biliary duct thrombi, splenomegaly, portal hypertension and comprehensive treatment further enhanced the development of liver cancer surgery. ④ In the 1990s, the concept of comprehensive therapy focusing mainly on surgery, the biotherapy strategy based on the rapidly developing molecular biology research and the study of liver transplantation for liver cancer are paid close attention.

The progress of diagnosis and treatment of primary liver cancer in recent years can be summarized as follows.

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Received 1998-10-18

EARLY DETECTION OF LIVER CANCER AND THE CHANGE OF THE CONCEPT OF SMALL LIVER CANCER

The methods for early detection of liver cancer include: ① men aged more than 35 years, with a history of hepatitis, and positive HBV or HCV, associated with cirrhosis or chronic hepatitis, should be recognized as a high risk population. Periodical monitoring of this population is a key step to find early liver cancers. ② AFP and B-US screening are, at present, the most sensitive, convenient and economical methods for detecting early liver cancers. ③ For the patients with low level AFP, AFP variant detection is helpful. As to the patients with negative AFP, other liver cancer markers can be used for the early diagnosis. ④ In combination with CT, MRI, CTA or DSA, B-US is of great benefit in the early diagnosis of liver cancer for both the nature of determination and localization ⑤ Fine needle aspiration for cytological assay or the diffusion way of the ethanol injected under ultrasonograph is also helpful for the establishment of the diagnosis.

The criteria for small liver cancer have not been standardized so far. In China, single nodular mass with the diameter or the sum of the diameters of two adjacent nodules less than 5cm was once regarded as small liver cancer. However, with the development of imaging tolls, the sensitivity is greatly increased. The liver cancers with the diameter less than 5cm are no longer regarded as small liver cancers clinically. The research in molecular pathology also showed that the great majority of liver cancers presented their biological features at the borderline of 3cm in diameter. Liver cancers with the diameter less than 3cm present the features of an early one, such as, growing largely in the swelling mode, cncapsulated, low incidence of vascular invasion and intra hepatic metastasis, diploid type of DNA contents and relatively slow growth. In contrast, the tumor with the diameter more than 3 cm has the capability of invasive growth and dissemination, the features of malignant biology and does not belong to the classical small liver cancer. In addition, as most of the liver cancer is homogeneous, tumor with more than two nodules has a higher probability of intra-hepatic metastasis. The concept that mononodular tumor with 3cm or less in diameter is a small liver cancer may be more suitable for the present liver cancer management and research. The early detection of liver cancer may significantly improve the efficacy of surgical

interventions. In our group[1], there was no surgical death in the 709 cases with the tumor diameter less than 5cm. The overall 1-year, 3-year and 5-year survival rates were 89.9%, 85.0% and 79.8%. Among them, of the 241 cases with 3cm or less in diameter, the overall one-year, 3-year and 5-year survival rates were 95.5%, 91.7% and 85.3% respectively. However, of the massive-sized liver cancers resected at the same period, they were 62.5%, 42.6% and 27.5% respectively.

THE HEPATECTOMY OF PRIMARY LIVER CANCER

At present, hepatectomy is still the treatment of choice for primary liver cancer. From 1960 to 1996[3], we performed 3932 cases of hepatectomy for liver cancer. Anatomical or extended hepatectomy accounted for 55.4% and local curative resection was 44.6%. The total mortality rate was 0.76% postoperatively, with 8.84% before 1997, 0.43% between 1978 and 1989 and 0.35% after 1990. The total 5-year survival rate was 36.1%, with 16.0% before 1977, 30.6% between 1978 and 1989 and 48.6% since 1990. One patient has survived more than 33 years. Comparing the survival rate listed above, we can tell that the efficacy of surgical intervention has been elvated significantly and rapidly since 1978. The main reasons are: the ability of early diagnosis was elevated; the renewal of some surgical concepts; the improvement of surgical techniques and perioperative management; and the development of comprehensive therapy postoperatively.

The pathological data from this group showed that 86.5% liver cancers were concomitant with cirrhosis or chronic hepatitis. The anatomical or extended hepatectomy might lead to a severe decompensated liver function. Therefore, the modality of liver resection drifted from a extended resection to an irregularly radical local one. Under the circumstance of chronic hepatitis or cirrhosis, the radical local resection not only increases the resectability, but also significantly decreases the surgical mortality rate and attains the same long-time therapeutic effects as the extended resection, or even better. The patients used to be subjected to conservative therapy when one or more complications presented, such as, jaundice, severe portal hypertension and esophageal varices with or without hemorrhage. With the accumulation of clinical practice, obstructive jaundiced patients resulting from involvement of hepatic hilus or cancerous thrombi invasion of biliary tract could undergo hepatectomy and the removal of biliary duct thrombi if hepatic cellular jaundice and other contra-indications could be ruled and most often, the jaundice disappeared gradually. To the patients with splenomegaly, hypersplenism and esophageal varices with or without hemorrhage, the hepatectomy can also be performed with splenectomy plus ligation of varices or portocaval shunts.

COMPREHENSIVE THERAPY FOR PRIMARY LIVER CANCER[4]

The comprehensive therapy was mainly focused on advanced liver cancers that were unresectable. But now, the concept of the comprehensive therapy for liver cancer is extended and includes ① the pre- and post-operative comprehensive therapy for resectable liver cancer to prevent the recurrence; ② palliative removal of incurably resected tumors followed by anti-cancer therapy in order to make the tumor shrink and prolong the survival time with tumor burden; and ③ comprehensive therapy for non-surgieal patients, with the hope of two-stage resection and long-time survival with tumor burden.

Comprehensive therapy includes surgical and non-surgical interventions. The former are hepatectomy, hepatic artery ligation (HAL), operative hepatic artery embolization (OHAE), drug delivery system (DDS), operative ethanol injection, microwave consolidation, laser gasification, freezing, etc. The latter are transcatheter arterial chemo-embolization (TACE), B-US directed percutaneous ethanol injection (PEI) or other drugs, radioisotopes and bio-agents, biotherapy, radiotherapy and traditional Chinese medicine.

Comprehensive therapy is so called with contrast to single method. Rational multimodality comprehensive therapy is superior to single method in terms of effects. The tetralogy method of surgical comprehensive therapy, which is the combination of HAL, OHAE, DDS and radiotherapy performed, in 603 advanced liver cancers in our hospital[2] showed that the rate of two-stage resection and one-year, 3-year and 5-year survival rate were significantly higher than that of single procedure (HAL or OHAE). The incidence of recurrence was only 7.4% in the 27 cases treated with comprehensive immuno-chemotherapy (cytokines plus low dose chemotherapy) after resection, while in control group, it was 32.0%. In 86 cases operated upon. DDS chemotherapy was adminiztered and the incidence of total one-year recurrence was 34.9%, while in hepatic artery group (n = 39), portal vein group (n = 26) and hepatic artery combining portal vein group were 33.3%, 34.6% and 23.6%, respectively. Non-surgical comprehensive therapy is indicative to almost all the unresectable liver cancer patients, with the methods of TACE and intra-tumor drug injection the most popular. In a series of 8 000 TACE cases, the 3-year survival rate was 13.9%.

The drugs was selected in the B-US directed local drug injection were absolute ethanol, ^{32}P radioisotope, OK432, TNF-α and IL-2. The 2-year survival rate in 700 patients receiving PEI was 80.0%, with a total of 3000 times treatment. In another group, 113 patients received TACE in combination with PEI, the tumors shrank in most patients (91.2%) at miscellaneous degrees, and the total 2-year survival rate was 81.6%. Among them, 11 of the 71 patients with monofocal large tumors received two[a2]stage resection after the tumors had shrunk and the two-stage resection rate was 14.28%.

Comprehensive therapy was not simply a random combination of miscellancous methods. If it was not properly combined, the therapeutic effects would be compromised. The design of the protocol should be case-specific. The model of the comprehensive therapy is multiple in literature. We propose two principles: ① attentions should be paid to the complimentary effects of each method. ② Avoiding the counteraction of the effects or the accumulation of side effects. At the same time, the toxic and negative effects of each method and its possible damage to the liver function should be paid enough attention. In addition, we stress the effects of traditional Chinese medicine in comprehensive therapy.

TWO-STAGE RESECTION OF PRIMARY LIVER CANCER[5]

In 1978, we reported a two-stage resection of a massive-sized liver cancer shrunk after HAL procedure. From then on, many reports followed and this procedure became a promising model for the unresectable large liver cancers. Surgical and non-surgical comprehensive therapies lead to the shrinkage of massive-sized liver cancers. At present, the documented methods for massive liver cancer shrinkage are: surgical comprehensive methods, such as HAL, OHAE and DDS, and non-surgical procedures, such as TACE, PEI, target therapy and radiotherapy. A rational combination of these methods makes some unresectable tumors resectable if they were successively employed. From 1974 to 1994, 649 patients received this therapy and 73 cases of them had their tumors resected with a resectability rate of 11.1% and no operative death. The 5-year survival rate was 61.5% postoperatively, with the longest survival being 17 years. The pathological data in this group showed that although the tumor shrank due to the comprehensive therapy, it was essential to remove the tumor because of the remnant living tumor cells. At present, though the reported two-stage resectability rates of liver cancer varies, the rates are still very low. The main causes of the low resectability rate are ① no general accepted criteria for tumor resectability. We propose that the two-stage resection is indicative only to the certainly unresectable tumors, otherwise, the one-staged removal is the first choice; ② rational employment of comprehensive therapy is crucial for the tumor shrinkage and ③ the unresectable liver cancers are recommended for non-surgical comprehensive therapy, such as TAE, PEI and guided chemoimmunotherapy as primary choice.

PROPHYLAXIS AND MANAGEMENT OF RECURRENCE[6]

The five-year recurrence rate in massive-sized liver cancers is 80%, while in small liver cancers, 40%-50%. The recurrence is most often found in liver, with few cases in bone, lung and brain or in abdominal cavity for the liver cancers ruptured before operation. The postoperative anti-recurrent comprehensive therapy, the early detection of the recurrent lesions and early management of the recurrent lesions are important steps to improve the therapeutic effects. Periodical postoperative follow-up is key to the early detection. We examine them with B-US, AFP and chest roentgenogram every 2-3 months. The patients with negative AFP are subjected to detection of other markers. The CT, MRI or CTA examinations are recommended to those highly suspected of recurrence. On the whole, the recurrence and metastases could be detected at subclinical stage. Comprehensive therapy is helpful in preventing the recurrence of liver cancer. The earliest recurrence happens within 2 months postoperatively, with the peak recurrence rate at 1-2 years. The recurrence after 5 years is rarely seen. Therefore, the anti-recurrent procedure should be given periodically in the 5 years after operation. Fine surgical manipulation to avoid medical dissemination, portal chemotherapy and suction of cancerous thrombi are all essential for prevention of recurrence. TACE, DDS, radiotherapy, immunotherapy and traditional Chinese medicine are given with detailed planning according to the condition of different patients. In recent years, we have employed immunochemotherapy, cytokines such as IFNs, TNF-α, TIL, CTL and some of them work well in anti-recurrence.

Reoperation is the treatment of choice for recurrent liver cancer. In this group, 123 patients received reoperation. The 1-year, 3-year, 5-year and 10-year survival rates after first resection were 99.2%, 71.4%, 53.2% and 19.1%. The 1-year, 3-year, 5-year survival rates after second resection were 83.5%, 38.2% and 19.6% while they were 94.7%, 44.9% and 25.0% after third resection. The reoperation of liver cancer is an effective

method for the improvement of 5-year survival rate and the establishment of reoperation concept has changed the idea that once the liver cancer recurred, it reached an advanced stage and was not fit for another operation. TACE and intra-tumor drug injection are indicative to those with poor liver function, hidden or multifocal lesions. In recent years, we performed PEI therapy in 109 recurrent cases with 0.7cm-15.2cm in diameter, averaging 4.6cm and the 1-year, 3-year and 5-year survival rates were 85.9%, 44.0% and 19.0% respectively. This procedure is easily performed and has the characteristic of faint side effects and damage.

CONSIDERATIONS FOR FURTHER IMPROVEMENT OF THE OUTCOME OF PRIMARY LIVER CANCER[8]

At present, several factors influence the prognosis of liver cancer clinically. They are ① whether small liver cancers detected early enough; ② pathological features of the liver cancer; ③ the curative degree of the resection; and ④ the efficacy of the anti-recurrent comprehensive therapy and the resectability of the recurrent lesions. For unresectable cases, the therapeutic effects depend on whether the comprehensive treatment is indicated and sensitive which will directly affect the survival and the two-stage resectability. To all of the liver cancer patients, the tolerance of the liver function to the long, successive traumatic therapy are the basis of therapeutic effects. Further improvement of therapeutic effects on liver cancer counts on the progress in basic liver cancer research. Recently, there were many progresses in the malignant biological features of liver cancer and new methods of biotherapy. The former includes clonal origin of liver cancer, oncogenes and enzymes related to the recurrence and metastasis of liver cancer and their mechanisms, glycoproteins and glycolipids research, the mechanism of the down-regulated immunity in liver cancer hosts and immune escape of liver cancer, induced differentiation of liver cancer, etc. The latter includes regimens that inhibit the recurrence and metastasis of liver cancer and angiogenesis inhibition therapy of liver cancer, specific active and passive immunotherapy, etc. Gene therapy and tumor vaceine technique are also developing rapidly.

It is controversial to the indications of liver transplantation on liver cancer. In advanced primary liver cancer, the recurrence after transplantation is unavoidable due to the vascular invasion and distal metastasis as well as immuno-suppressive agents used. On the contrary, the therapeutic effects of liver transplantation on small liver cancer combined with severe cirrhosis are corroborated. Comparing the therapeutic effects of hepatectomy and liver transplantation (60 cases each). Bismuth[9] concluded that the 3-year survival rates were almost the same, while the 3-year tumor-free survival rate was higher in liver transplantation group than in hepatectomy group. As to the small liver cancer (mononodular or binodular, with the diameter less than 3 cm), the results of liver transplantation were even better. Selby *et al*[10] showed that the overall 5-year survival rate in 105 unresectable cases of different stages that received liver transplantation was 36%, of whom the 5-year survival rate for one to three stage was up to 52.1%, while in stage four, it declined to 11%. They concluded that liver transplantation was fit for liver cancer in early stages ($\leqslant$2cm, no vascular invasion and no distal metastasis). However, we still could not regard liver transplantation as a routine therapeutic method due to high incidence of liver cancer, liver donation shortage and high cost.

With the accumulation of 30 years of clinical study, especially the research work during the past decade, we extended our knowledge in its biological characteristics, its clinical features and its diagnosis and treatments. The great efforts should be made for further improving the overall therapeutic results of liver cancer.

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Conference Proceeding

Strengthen international academic cooperation and exchanges: prospects in the 21st century: Summary of the First World Chinese Congress of Digestion

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Subject headings academic cooperation; digestive neoplasms;digestive diseases

The First World Chinese Congress of Digenstion was held in Beijing from October 20 to 22, 1998 in the beautiful capital city of Beijing. The specific aim of this meeting is to summarize and exchange the experience of modem and traditiional digestive medicine, and to enhance academic exchanges and cooperation among the Chinese and all other scientists in the world. The congress includes extensive topics, such as the telomerase activity in hepatic carcinoma tissues, acupuncture and moxibustion expression of cellular factor related genes in ulcer colitis in rats antifibrosis by tetrandrine, and *Helicobacter pylori*, gastric carcinoma etc. More than one thousand participants from 12 countries, including the United States, United Kingdom, Australia, Japan, Canada, Israel, South Korea, etc attended this magnificent meeting. Nineteen hundred and eighty-six abstracts were submitted to the meeting and from which, seven hundred abstracts were accepted. Some distinquished experts were invited to make special lectures at the plenary sessions (*WJG*, Supplement 2, 1998) and 81 doctors and researchers made presentation at the symposia mainly on the following areas.

DIGESTIVE NEOPLASMS

Esophageal cancer

Alteration of *p*19 mRNA expression in esophageal cancer tissue from patients at high incidence area in northern China was reported by Qi *et al*, from Henan Medical University. RT-PCR was used to measure the expression of *p*19ARF, *p*53 and *p*21 in 19 pairs of frozen normal esophageal and tumor samples. The cycle number for each pair of primers was fine-tuned to limit the amplification to a linear range. PCR products were then resolved on 2% agrose gel. The densityh and area of each band was measured using image-pro-plus 1.3 software. The relative expression level of each gene in tumor and normal tissues was calculated using the house-keeping gene GAPDH as an internal control. In the total of 19 tumor samples, 8 (42%) had at least a 3-fold decrease in *p*19 ARF but with no decrease in p53 expression, 5 (26%) had significantly decreased expression of *p*53 but had normal expression of *p*19ARF, only two sample (11%) had decreased level in both *p*19ARF and *p*53 expression. The results suggest a negative correlation between the alterations of these two genes in the esophageal tumor. The relative expression level of *p*21 in *p*19ARF negative sample (0.78±0.16) was about half of that in *p*19ARF positive samples (1.63±0.22). The results support the hypothesis that *p*19 inactivation contributes to esophageal tumor progression and follows the same pathway as *p*53 and *p*21. The cyclin-dependent kinase inhibitor *p*16 and *p*15 play important roles in the regulation of the cell cycle, and have been found to have tumor suppressing roles in a variety of types of cancer. It has been shown that *p*16 aberrant methylation and *p*15 homozygous deletions were frequently involved in human esophageal squamous cell carcinoma (ESCC). The this study examined the impact of such molecular alterations on the expression of these genes. Jiao *et al* (Henan Medical University) measured the mRNA level of both genes in 21 frozen ESCC specimens using semiquantiative RT-PCR. Nineteen cases were observed at a low basal level of *p*16 expression (0.11±0.07, expression units normalized by housekeeping glyceraldehyde-3-phosphate dehydrogenate gene as internal standard) in the normal epithelia adjacent to the cancer tissues. Among the 19 cases, only 5 showed a significant elevation of *p*16 expression (>3.2 folds) in the tumor, whereas the remaining 14 showed either a slight increased (1-2 folds), or decreased *p*16 expression compared to normal, whereas 11 had

Editorial Board of World Journal of Gastroenterology, Beijing 100023, China

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Received 1998-10-24

only a slight increase (1-2 folds), or decreased *p*16 expression compared to normal. In the 5 cases where *p*15 was already activated ($P>0.5$) in the adjacent normal epithelium, 4 of them had similar or a slightly lower expression level, but one had a great decrease in *p*15 expression (<1% of the normal level). For intact *p*16 and *p*15 genes, which encode cell cycle regulators, significant increase of their expression on expected in the cancer cells as a response to accelerated cellular proliferation. The findings from Bai et al (Henan Medical University) indicate that the occurrence of hMSH2 protein expression is associated with the cell cycles and related to PCNA expression, implying that the hMSH2 protein is expressed as a guardian in DNA-Synthesizing cells.

To observe the morphological changes of the cell apoptosis of esophageal carcinoma cell line EC8712 induced by arsenic trioxide, Shen *et al*, Shantou University Medical College studied the morphology on apoptosis of esophageal carcinoma cell line induced by arsenic trioxide EC8712 cells routinely cultured in 199 medium and acted under 3μmol As_2O_3 harvested after 72 hours, and then HE-stained, TUNEL labeled and examined by transmitting electron microscope and flow cytometry. When adding As_2O_3 (3μmol) to EC8712 cells for 72 hours, many apoptotic cells appeared. Under light microscope, two kinds of apoptotic cells were seen after H-staining. One was condensed, small-sized and rounded in shape with nucleus dented. The other cells had eosin stained cytoplasm, chromatin agglutinated and the nucleolus existed. The nuclei of the apoptotic cells were positive by labelling with TUNEL kit. The apoptotic peak was identified by flow cytometry. Under electron microscope, two kinds of apoptotic modes were also seen. At early stage of apotosis, the chromatin of the nucleus agglutinated to pieces and the organelles in the cytoplasm preserved complete. At the second stage, following the change of chromatin, the nucleus became round and full with small pieces of chromatin stickled to the nuclear membrane, while the large clumps of chromatin made the nucleus look like a wheel or a crescent mass. Most of the apoptotic cells showed disintegration of nuclear membrane, from which the chromatin flew out. At the final stage, the apoptotic cells showed degeneration or nerosis. Apoptotic bodies were easily seen which were dense, piece-like or ball-like, naked or capsulated. In the other mode of apoptosis, pyknotic cells showed cell shrinkage, cytoplasm condensation, high electron density and gradual solidification of cell nucleus. CT scan in esophageal carcinoma is reliable and accurate. The tumor center should be taken on simulation in order to encompass the whole tumor by the 80%-90% isodose curves. He *et al* (lingi Cancer Hospital, Shandong) investigates the protective effect of radiated auto-blood transfusion on radiation. The incidence of acute radioactive esophagitis was 12.1% in study group and 60.6% in control group ($P<0.01$). The average dose of radiotherapy causing acute radioactive esophagitis was 4050±609 (cGY) in control group ($P<0.01$). Significant change of IL-2 and T-cell subgroups was seen in study group. Low dose radiation can stimulate the body's immune function, through which the threshold dose of radiotherapy increases without damaging normal tissues.

Stomach neoplasm

To diagnose the alteration of oncogenes and tumor suppressor genes in gastric carcinogens, Cen *et al* (Affiliated Hospital of Qiannan Medical College) researched on *P*53 gene mujtation of biopsy samples from stomach cancer patients. The mutation rate of *P*53 in exon 5-8 was 60.0% (18/30). Point mutation of *P*53 was found at both early and advanced tumors. In contrast amplification of oncogenes and loss of tumor suppressor genes were correlated with poorly differentiated and metastatic tumors.

The induction of apoptosis by cisplatin (CDDP) in gastric carcinoma cell line for demonstration of a human gastric carcinoma with CDDP was investigated by Xin *et al* (Fourth Military Medical University). A human gastric carcinoma cell line SGC-7901 was cultured in full medium with various doses of cisplatin for different hours. The treated cells were examined under light microscope and transmission electron microscope (TEM). Cell cycle analysis was performed in flow cytometry (FCM). After treatment with CDDP, the cells became smaller and condensed. Their chromatin changed to periphery of the nucleus. Apoptotic bodies were observed. There were apoptotic peas in cell cycle analysis on FCM. There were apoptotic peaks in cell were 9.3% and 14.9% after being treated. CDDP might induce apoptosis in the gastric carcinoma cell line SGC-7901 that led to the death of the cancer cell. The data of Beijing Medical University (Cao *et al*) show that antisense RNA to bcl-2, not only can induce apoptosis, but also reverse the biological behavior of MGC-803 cells. This would be a potential application to the gene therapy for stomach cancers. Preoperative cimetidine application can restore NK cells, which may be beneficial to reducing recurrence and metastasis (Li *et al*, Hebei Medical University). Ma *et al* (Third

Military Medical University) detected the occurrence and development of precancerous lesion of residual gastric mucosa and their relationship to gastric cancer in the gastric stump. The conventional Billroth gastrectomy is closely associated with the lesion of the residual gastric mucosa. The common manifestations of gland atrophy and proliferative lesions of the residual gastric mucosa are the important bases of the precanserous lesion, which should be paid more attention to in clinical practice. Shen *et al* (Nantong Medical College) observed the therapeutic effect of compound Shenqitang decoction on gastric adenocarcinoma after gastrectomy, and studied it inhibitory effect on gastric adenocarcinorma induced by MNNG in Wistar rats. The results shown that it has good therapeutic results in combined operative treatment of gastric carcinoma. Colonic neoplasms periphera blood (PB) T-lymphocyte subsets and natural killer cytotoxicity (NKCC) were measured in 43 patients with colorectal carinoma (CRC) pre- and post-operatively by using the APAAP and LDH release methods respectively. The CRC patients were still in immunodepressive state in the first 2 weeks after operation, and the immunotherapy can improve the preoperative cellular immunofunction, and shorten the perioperative immunodepressive period (Liu *et al*, Taian Central Hospital). Xiao *et al* (Central Hospital in Jianghan Oil Field) explored the therapeutic effect of chemoembolization in hepatic metastases in colorectal carcinoma. Forty patients underwent chemoembolization of metastatic liver lesion from colorecial carcinoma. Selective angiography of the hepatic artery was performed to identify the feeding vessels of the metastatic lesion. The injected chemoemulsum consisted of 100 mg 5-fluorouracil, 10mg mitomycin c and 10 ml lipiodol ultra-fluid in a total volume of 30 ml. Gel foam embolization was then followed until stagnation of blood flow was achieved. Patients were evaluated for response, overall survival, and side effects. Overall median survival time from date of first chemoembolization was ten months. Median survival time of cirrhotic patients with class A and B by Child-Pugh classification was 24 and 3 months, respectively. The difference was significant ($P < 0.01$). Patients with metastatic disease confined to the liver did better than those who also had extrahepatic disease, with median survivals of 14 and 3 months, respectively ($P < 0.02$). The median survival of patients with hypervascular metastases was longer than that of patients with hypovascular metastases. The most common side effects were transient fever, abdominal pain and fatigue. Three patients died within one month from the procedure. The therapeutic effect of systemic chemotherapy in hepatic metastases of large intestinal carcinoma was not satisfactory and there were more side effects, whereas the therapeutic effect of selective chemoembolization was promising and there were fewer side effects. Selective chemoembolization may be an effective first-line therapy in hepatic metastases of large intestinal carcinoma. Gao *et al* (The Harrison International Peace Hospital) reported that after abdomino-perineal resection of rectal cancer (Miles), greater omentum was cut off and retroperitoneal tunnel was performed. According to the ways of greater omentum into pelvis, the tunnels had three ways (left, middle and right). Left way reaches pelvis through retroperitoneal tunnel in descending colon side dish, 6 cases; middle way reaches pelvis through retroperitoneal tunnel behind colon and on the left of spine, 35 cases; and right way reaches pelvis through retroperitoneal tunnel in ascending colon side ditch, 9 cases. Middle way is the best, which has a short tunnel, is situated below abdomenal incision, convenient and easy on practice. Packing in presacral space with the pedicle greater omentum transplantation has better effects on promoting the primary healing of the perennial wound, with extensively clinical application value.

Liver and pancreatic neoplasm

Hepatic arterial branch supplying hepatocellular carcinoma (HCC) has a lower impedance than the branch not supplying HCC (Wang JG & Pan LL, Shantou Central Hospital). To study the blood AFPmRNA in the patients with distant metastasis of human HCC using nested reverse transcriptase polymerase chain reaction (nested RT-PCR) and its significance, 93 blood samples from human HCC were examined by nested RT-PCR to find out AFPmRNA by Liu *et al* (Second Military Medical University). AFPmRNA was detected in 21 blood samples from 72 human HCC (40.28%) without distant metastasis. AFPmRNA (100%) was detected in all HCC patients with distant metastasis. AFPmRNA can be used as a distant metastasis marker of HCC. Portal vein chemotherapy combined with 0.25MPa HBO can significantly reduce the tissue impairment and oxygen radical after resection of HCC. These findings may indicate that hyperbacicc oxygenation plays a positive role in combined treatment for HCC (Li *et al*, Fujian Medical University). Blood AFPmRNA and AFP detection is useful in predicting relapse or distant metastasis after surgery in HCC patients (Zhang *et al*, Second Military Medical University). Para-HCC

specimens (24 cases, Group A) and noncancer cirrhosis (33 cases, Group B) were all tested by *in situ* terminal end labeling (ISEL), HBsAg immunohistochemistry and HE analysis. ISEL (+) intensity was divided into 4 grades. The results were compared between the two groups. The positivity rates and positive intensity of ISEL in Group A were significantly higher than that in Group B ($P < 0.01$). The positive cell nuclei tended to scatter just near the septa and portal tracts. The majority of Group A are of static portal cirrhosis, while Group B also included cirrhosis of chronic active hepatitis and chronic severe hepatitis. Inflammatory cell infiltration was more evident in Group B that in Group A ($P < 0.05$). The HBsAg(+) rates of both Groups A and B are very high. There were no correlation among ISEL and HBsAg, proliferation and dysphasia of hepatocyte. About half of the hepatocytes in one case of Group A underwent apoptosis identified by both ISEL and HE (Lian *et al*, Shantou University Medical College). The detection of TGF-β1 and PCNA expressions in primary hepatocarcinoma tissues may be useful in identifying and judging the tumor-differentiation and prognosis (He *et al*, China Medical University). The ras oncogene and *p*53 anti-oncogene expressions of 55 pancreatic paraffin-embedded specimens, including 32 carcinomas, were studied by immunohistochemistry ABC method. Twelve specimens taken from the normal pancreatic tissue near the tumor transaction margin, 7 specimens of pancreatitis and 4 specimens of normal pancreas were compared.

The positive expression rate of *ras* oncogene and p53 antioncogene was 71.9% and 28.1% in 32 cases of pancreatic carcinomas and it is higher than that of the pancreatitis and normal pancreatic tissue near the tumor transected margin ($P <0.05$). The ras and *p*53 gene expression was not significantly related to sex, age, site, size and incipient symptoms ($P > 0.05$). The *p*53 antioncogene expression was related to tumor staging and grading ($P<0.05$). The tumor mass with negative *p*53 gene expression usually had a higher respectability ($P < 0.05$), and its positive expression usually associates with lymph node metastasis ($P<0.05$), and worse prognosis. It also provided an important guidance to choose the methods of treatment. Sun *et al* (Guiyang Medical College) suggested that the *ras* and *p*53 gene expression could be used to evaluate the pancreatic carcerous biological behaviour, and it might aid the diagnosis and treatment for pancreatic carcinomas.

GASTROINTESTINAL DISEASES

The poor living condition is the sources of the *Hp* infection, and it is the main pathogenetic factor of PU and upper gastrointestinal tumor. Kang *et al* (People's Hospital of Liulin County) investigated and summarized incidence of upper gastrointestinal diseases in Liulin County. A total of 3142 patients were had *Hp* tested by urease and pathological tests. Gastric ulcer was found in 287 cases, duodenal ulcer in 245 cases, and esophageal cancer in 241 cases. The rate of *Hp* infection was 98% in gastric ulcer, duodenal ulcer and cancer of the stomach. The ratio of GU to DU was 1.17:1, including 672 PU cases, and 414 cases of malignant tumor. The incidence of PU, esophagus cancer and stomach cancer was found to be increasing. The incidence of GU in males was much higher than that in other areas reported, possibly due to living conditions and dietary habits. One hundred patients with chronic atrophic gastritis were treated with Wuji capsule. Of them, 42 were mild, 38 moderate and 20 severe atrophic gastritis and 41 and 13 asccompanied with intestinal metaplasia (IM) and degree I dysphasia (Dys), respectively. The clinical manifestations were stomach pain (86 patients), fulless of abdomen (72), anorexia (90), eructation (34) and bitterness of the mouth (20). After treatment for three months the improvement of patient's symptoms, atrophy of gastric mucosa, IM and Dys were annualized. After 3 months with Wuji capsule treatment, 7 patients were recovered, 48 veryu effective, 34 improved, and 11 infective. The total efficacy waqs 89%, and 5 unchanged. Of the 38 patients with moderate atrophic gastritis, 17 development mild atrophic gastritis, 14 superficial gastritis, 3 became normal and 4 unchanged. Of the 20 severe atrophic gastritis, 10 turned into moderate atrophic gastritis, 8 superficial gastritis and 2 had no changes. Of the 26 patients with mild IM, IM disappeared in 20, and 6 had no changes. Of the 8 patients with moderate IM, IM disappeared in 1, 4 changed into mild IM, and 3 had no changes. Of the 7 patients with severe IM, 2 changed into moderate IM, 2 mild IM, and 3 had no changes. Of the 13 patients with degree I Dys, Dys disappeared in 7, and 6 had no changes. Hao *et al* (China Medical University) investigated the effects of different kinds of Bupleurum and Citrus on gastrointestinal motility. Choosing the two main wareties of Bupleurum and Citrus, B. Chinese DC. (B. Cdc), B. Scorzonerffolium Wild (B.sW) and Citrus aurantium (Ca), Citrus sinensis (Cs), as the test drugs, we compared the effects of 4 drugs. Different mixtures of Bupleurum and Citrus and different dosage of the mixtures on mice gastrointestinal motility with Blue Dxtran 2000 as a

marker in the gastrointestinal tract. B. cDC. and Ca had obvious enhancing effects on the gastric emptying function and small intestinal propulsion function, while the effect of B.sW and Cs had no difference with negative control group ($P > 0.05$). The effects of the gastrointestinal motility proved to be more significant than single drug and the mixture of the above two herbs decocted respectively. Li *et al* (China Medical University) investigate the influences on gastric emptying and small intestine transportation of 6 formula compositions combined with atractylodis ovatae rhizoma (AOR), Magnoliae Cortex (MC), Arecae Pericarpium (AP), Amomi SemenSeu Fructus (ASSF), Galli Gigerii Endothelium (GGE), Massa Medicata Fermentata (MMF), Hordei Frutus Germinalus (HFG) and Caratecgi Endocarpiumet Semen (ACES). The decoctions of ASSF, GGE, MMF and HFG, MC, AP, MMF and HFG; MC, AP ASF, GGE, MMF, HFG; and the decoction of MC, AP, MMF, HFG, ACES and APR can improve the gastric emptying function. The decoction of ASSF, GGE, MMF and HFG can also promote the small intestinal transportation function. Zhang *et al* (Youhong Chinese Medicine, Huinong) analysed the therapeutic effect of Jieyu Yuyang San, Xiaqi Xiaoshi Yutong San, Yangyin Yuyang Zhentong Wan on three kinds of peptic ulcer. After the whole course treatment, 382 affective ulcer patients were recovered, 120 had evident effect, 204 improved, and 14 ineffective, with a total effective rate of 98%. A total of 450 diatary ulcer patients were recovered, 240 very effective, 108 improved, and 32 ineffective, with a total effective rate of 96.2%; and 301 mixed ulcer patients were recovered, 209 very effective, 107 improved, and 33 ineffective with a total effective rate of 95%. Symptoms disappeared in 2121 patients, and 79 patients ineffective with a cure rate of 77.6%. Before treatment, the degree I, II, III peptic ulcer was found in 720, 830 and 650 patients and 14, 32 and 33, respectively after treatment. Of 157 cases of liver cirrhotic ascites, 57 cases had upper gastrointestinal bleeding, and 100 cases had no bleeding. Complications uncluded hypersplenia, gastric ulcer, spontaneous peritonitis, hepatic coma and poor renal function. Complications in positive bleeding group were compared with negative bleeding group as controls. The positive rates of poor renal function and hepatic coma in positive bleeding group were significantly increased as compared to that in controls ($P < 0.01$). The positive rates of complications with upper gastrointe stinal bleeding in ascitic liver cirrhosis were higher than non-bleeding. In order to investigate the cause and the position of bleeding with cirrhotic ascities and seatch for therapeutic methods, emergency endoscopy was performed (Li *et al*, Hainan Provincial People's Hospital). Assessment of disease outcome in a large inception cohort of patients with IBD showed that the majority had symptomatic improvement over a four-year period after diagnosis and mortality from IBD related causes was low. In Europe, with present medical treatment, medium-term outcome of IBD appears favorable. The plasma concentration of nitrite/nitrate (stable end products of NO, standing for NO) and molitin of 18 patients with UC and 11 control subjects were respectively measured with Cadmiun-reduction chromatgraphy and development process (Greiss) and RIA (Radioimmuno assay). The concentration of plasma NO and MTL in UC groups were significantly higher than the controls ($P < 0.01$, $P < 0.05$, respectively). The concentration change of plasma NO in UC group significantly correlated with the change of MTL ($P <0.05$); but there was no significant correlation in the control group ($P < 0.02$). Nitric oxde and molitin were both involved in the pathophysiologic process of ulcerative colitis. Moreover, there may be some positive interactions between NO and MTL in the pathogenesis of UC (Wu *et al*, Fujian Medical University). Hu *et al* (461st Hospital of PLA) observed the therapeutic effects of ulcerative colitis managed by integrated traditional Chinese medicine (TCM) and western therapy and compared with conventional interventions solely. The results were investigated 2 weeks afterward 39 cases cured (81.3%), 8 improved (16.7%) and 1 case ineffective (2%), i.e. 98% total effective rate in Group I. Comparatively 27 cases cured immediately (64.3%), 9 improved (21.4%) and 6 ineffectiveness (14.3%). With a total effective rate of 86.9% in Group II. Significant differences were found statistically between the two groups ($P < 0.01$). CD and UC are two forms of intestinal inflammation with possible common genetic predisposition and may be part of a spectrum, rather than two distinct disease. Induction may be non-specific. Genetic susceptibility and uptake of bacterial poroducts perpetuate inflammation. Genetic and environmental factors are cirtical, but neither alone is sufficient. Progression and resolution of CD and UC are dependent on the balance of pro- and anti-inflammatory mediators. Homeostasis or chronic inflammation depends on the balance between inflammatory luminal constituents and protective mocosal factors. Specific therapy directed at an immunoregulatory defect or an inciting agent could alter the disease course. Current

therapies, such as glucocorticolds and 5-aminosalicylic acid (5-ASA), inhibit concentrations of interdependent, soluble mediators of inflammation, which may amplify one another or have parallel effects. In remains, however, to define whether targeting multi-inflammatory actions or a single key pivotal process is a better therapeutic strategy. Thye type of new drugs being developed include conventional pharmaceuticals, receptor antagonists-agonizes, enzyme inhibitors, bio-engineered compounds (monoclonal antibodies, chimerical-targeted toxins, receptor legends-soluble receptors), and gene therapy.

LIVER, BILIARY AND PANCREATIC DISEASES

In cold weather, upper gastrointestinal hemorrhage is common in the patients with liver cirrhosis. The mechanism is that the change of temperature affects the redistribution of blood of the human body. When the weather temperature drops, the effective blood circulatory volume of shallow tissues is reduced to some extent, while that of deep tissues is increased relatively. This will raise the pressure of portal vein system and its collateral circulation. Guo *et al* (Second Military University) analyzed the relationship between cr1 genetic density polymorphism on erythrocytes and ability of erythrocytes adhering tumor cells in different groups, such as normal people, patients with HBV infection, liver cirrhosis and liver cancer. In the same population, the ability of HH type erythrocyte adhering tumor cells was significantly higher than that of HL type erythrocytes. The ability of HL type erythrocytes adhering tumor cells was significantly higher than that in LL type erythrocytes. In the same cr1 genomic type population, the ability of erythrocytes adhering tumor cells of normal people was significantly higher than that of patients with HBV infection and pathents with liver corrihosis and liver cancer. Zhang *et al* (General Hospital of Jinan Command Area) established liver injury model induced by ConA in Kunming mice. ConA was administered to Kinming mice via tail vein. The model was dese dependent; the histopathological examinations of liver specimen showed the T lymphocytes infiltration in portal areas, spot necrosis and piecemeal necrosis. With the inhibition of T cell activation by cyclosporine A (CSA), liver injury and infiltration of lymphocytes were not seen. Huang *et al* (Fujian Medical University) explored the clinical significance of serum type III procollagen (PC III), laminin (LN), prolidase (PLD) and type IV collagen in patients with liver diseases. Serum levels of LN, PC III, IV-C and PLD were helpful in clincal diagnosis of patients with liver cirrhosis and in judgment of developing tendency in patients with chronic HBV infection. Combined determination of PCIII and LN can elevate the specificity in the diagnosis of cirrhosis. The level of γ globulin can reflect the pathology of the liver, the level of serum cholinesterase can reflect the synthetic function of the liver and is negatively related to the damage of the liver (Zou *et al*, Chinese PLA 302 Hospital). Dan *et al* (Chinese PLA 302 Hospital) reported that isolates of HCV genotype 1b between China and Japan share high similarity in NS5A nucleotide sequence. Variation in the NS5A region between amino 2209 to 2248 failed to predict IFN response in Chinese patients infected with HCV genotype 1b. Xu *et al* (Shuang Ya Shan General Hospital) reproted that estradiol and HCG are related to the formation of gallbladder cholesterol stone. Sixty rabbits were randomly divided into 6 groups, in 4 of which (groups E2, P, T, H) estradiol, progesterone, testosterone and HCG were administered separately, and normal saline and refined oil were given to the other groups (C_1, C_2) as control. The animal were sacrificed after 6 weeks. The blood, bile, gallbladder, bile duct, liver and gallstones were assayed. The gallstone formation rate was 90% in group E_2, 50% in group H and 10% in group T. No gallstone was formed in group P, C_1 and C_2. Most of the gallstones were found in female animals, only in 4 male rabbits of group E2. The composition of stone was mainly cholesterol (Wang *et al*, Anhui Medical University). Wang *et al* (Guiyang Second People's Hospital) studied the relationship between the estrogen, blood lipids and cholelithiasis. Serum estradiol (E_2), progesterone (P), total cholesterol (TC), triglyceride (TG) were tested in 104 patients (Group A) confirmed to have cholecystolithiasis by B-mode ultrosonography and cholecystostomy and the results were compared with that of 54 normal persons (group B). Serum E_2 and P levels of the men in group A were remarkably higher than those in group B (P<0.05-0.01). Serum E2 levels of the women of child-bearing age were not different between groups A and B (P>0.05), but P levels of group A were higher than that of group B. Serum levels of E2 or P of menopause women in group A were all markedly higher than those of women in group B (P<0.01). The ratio of E2/P of women in group A was siguificantly lower than those in group B (P < 0.001). Serum levels of TG, TC and the ratio of TG/TC in persons of group A (either men or women) were all higher than those in group B (P <0.01). Estrogen and lipid metabolism of

cholecystolithiasis patients are disordered. The role of oxygen free radical (OFR) and other inflammatory mediators in acute necrotized pancreatitis (ANP) was studied by Wang *et al* (Inner Mongolia Medical College). Oxygen free radicals were involved in the aggravation of ANP and were associated with the increased of serum endotoxin and PLA2. Those mediators were positively correlated with severe multiple organ damage. The results also suggested that IL-2 could inhibit the overexpression of OFR and endotoxin, and reduce the incidence of multiple organ damage in ANP. TNFα mRNA plays an important role in ANP progression and somatostatin and growth hormone may be the effectual treatment to prevent the development and progression of multiple organ dysfunction syndrome in acute necrotized pancreatitis (Zhang *et al*, Shanghai Medical University). Zhong *et al* (Zhongshan Medical University) suggested that patients with acute pancreatitis have significantly different changes of platelet formative property from acute hemorrhage and necrotized pancreatitis, which indicates the severity of the disease. Pt has no significant change, but platelet activity was increased after SS treatment. Qin *et al* (Luoyang Second People's Hospital) evaluated thye curative effects of Octretide (Oct) on acute pancreatitis. Oct was used to treat 38 cases of acute pancreatitis, and 59 patients were treated as the control group by non-octreotide. Before and after Oct was injected, serum amylase and pancreatic fluid amylase were analyzed quantitatively for the two groups, and the incidence of complications were also compared among these patients. Oct was found to ampeliorate the clinical symptoms and signs and decrease the occurrence of complications.

HELICOBACTOR PYLORI

Helicobacter pylori (*Hp*) is a gastric pathogen strongly implicated in the causation of gastritis, ducdenal ulcer, gastric ulcer, gastric cancer and gastric lymphoma. Almost half of the world's population or 2 billion people are *Hp* infected, making it the commonest chronic infection in men, and an important global health problem. There are several striking differences in the pattern of *Hp* infection and gastroduodenal diseases between countries of the Esat and West, including: *Hp* presence and characteristics; disease patterns; and host differences. These differences do not occur on the basis of genographic boundaries, but are the outcome of genetic and environmental factors in the respective populations. Strategies for the management of *Hp* infection in Asia must take these factors into account. These differences and their implications for clinical management and health care policies in Asian countries were presented by Dr. Yeoh Khay Guan (National University Hospital, Singapore). Xu *et al* (Harbin Medical University) demonstrated that CCK-8 could antagonize the effect of morphine which inhibited the potentiation of Ache on the electrical and mechanical activities of ran duodenum *in vitro*, whereas devazepide could reverse the anti-morphine effect of CCK-8. It is suggested that the antagonistic effect of CCK-8 on morphine should be mainly mediated by CCK-A receptor, thus providing a new clue for the clinical treatment of disturbances in intestinal movement function. *Hp* infection is closly related to DU occurrence and can lead to antral gastritis. Owing to *Hp* antral gastritis, antral D cells in patients with DU decrease in number and SS synthesis (Zheng *et al*, China Medical University). Han *et al* (Chinese PLA Institute of Genetic Diagnosis) cloned the 5'-end of cagA (854bp) into the expression vector pBV220 and transformed DH5α with the plasmid pBV220/ fcagA, in which a single CagA fragment (FCagA) was produced when the temperature reached 42℃. After being renaturated, FcagA was purified by anion exchange and sephadex G-100 chromatography. The FcagA had a relative molecular weight of 38 000. With the prepared FCagA, coloidal gold, and immunogold, they established the dot immunogold filtration assay (DIGFA) to detect anti-CagA antibody in serum. FCagA had the similar antigenicity as CagA. The test of DIGFA took only a few minutes and could also be done for one or more persons with no need for special equipment. Compared with EIA, DIGFA had the sensitivity of 96.8%, and the specificity of 98.5%, when the sera of 262 cases were tested. One hundred and sixty-six patients completed all the study. The eradication rates were 84.2% in group A and 72.2% in group B ($P < 0.05$). There was no significant difference in both the *Hp* eradication rate and healing rate of the ulcer patients between the two groups. More side effects occurred in group B than in group A, which still could be tolerated by the patients. The cost of group A is higher than that in group B (RMB 820.78 *vs* 418.04). Considering the effectiveness and cost, OCA therapy is more suitable for gastritis patients. For ulcer patients, RTA therapy is as effective as OCA therapy (Wang *et al*, Shanghai Zhongshan Hospital). Gastrin (Gas) and somatostatin (SS) of gastric mucosa and blood in patients with *Hp* positive group were significantly higher than *Hp* negative group and became normal after *Hp*

eradication. The SS contents in *Hp* positive group were significantly lower than *Hp* negative group and became normal after of the *Hp* eradication. On the other hand, Gas and SS contents of the mucosa significantly altered with chronic and active inflammations. Zhang *et al* (China Medical University) studied the sensitivity, specifity and clinical applicatia of 14C urea breathing thes of *Hp* infection. All the 150 cases (40 cases of chronic gastritis, 30 cases of gastric ulcer, 50 cases of duodenal ulcer, 20 cases of gastric carcinoma, 8 cases of polypous gastritis, 2 cases of portal hypertensive gasteopathy) were examined by fibrogastroscopy and confirmed by biopsy pathology, 15 cases of duodenal ulcer and 5 cases of gastric ulcer were treated with PPI therapy for 1 month, then a comparison between the pretreatment and posttreatment was made, ^{14}C urea was calculated by scintillators. The results showed that the *Hp* infection rates of chronic gastritis duodenal ulcer and gastric ulcer and gastric carcinoma had no statistical difference with the method of ^{14}C-UBT; there was no difference in ^{14}C-UBTY radioactivity value between chronic gastritis and duodenal ulcer; the incidence of chronic gastritis accompanied with gastric mucosal erosion atrophy and enterometaplasia was significantly higher than that of simple chronic gastritis ($P<$ 0.05); after one-month bactericidal treatment, the bactericidal rate reached 100%. In conclusion, this brief glimpse into the science and practice of gastroenterology in the next century offers us a mixed perspective, one of an ever-widening disparity between rising opportunities in the one hand, and restrained resources on the other. We are afraid that uless this serious dilemma will be resolved early in the next century, the practice of gastroenterology and the quality of health care will cut expenses by voluntarily reducing our dependence on technical procedures and expensive equipment, and by avoiding use of only marginally effective medications and surgical interventions. These, we believe, will be painful adjustments for the medical establishment, but they must be faced in the coming century.

Original Articles

Coexistence of *Helicobacter pylori* spiral and coccoid forms in experimental mice

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Subject headings *Helicobacter pylori*, antibodies; ELISA; spiral form; coccoid form; mouse

Abstract

AIM To infect mice with *Helicobacter pylori* and detect immune response against two form of *H. pylori*.

METHODS An isolate of *H. pylori* obtained from a patient with gastric cancer was used to infect mice. Fifty mice were divided into eight groups. Two groups served as negative control without any inoculation and internal negative control with 0.5M NaHCO3 and brain heart infusion (HBI), respectively. Mice in each experimental group were first inoculated with 0.5M NaHCO3 and then *H. pylori* suspension for 3 times at a 2-day interval. Mice from controls and infectious groups were sacrificed at a weekly interval postinfection. Gastric samples were trimmed, inoculated onto chocolate blood agar and then incujbated in microaerophilic atmosphere at 37iæ for 14 days. Sera were examined for immunoglobulins against *H. pylori* spiral and coccoid antigens by ELISA.

RESULTS After inoculation *H. pylori* was isolated in one mouse from one week postinfection. No *H. pylori* was detected in control mice. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with NaHCO3 and BHI and 77% (23/30) mice infected with *H. pylori*. The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The mice showed immune responses to both *H. pylori* spiral and coccoid antigens one week after infection with *H. pylori*. The peak mean absorbances of antibodies against spiral and coccoid forms were four weeks postinfection which showed 6 and 18 times higher than that of negative control group respectively ($P<0.01$).

CONCLUSION Spiral and coccoid forms of *H. pylori* coexist in experimental mice studied.

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Received 1998-11-09

INTRODUCTION

Helicobacter pylori colonizes stomach of human being and causes gastritis and peptic ulcer[1]. It has been reported that this organism exists in two forms, spiral form and coccoid form[2,3]. Many investigations are being performed on whether coccoid form is degenerative or viable. Hua and Ho[3] reported that similar to the exponential cultures, ageing coccoid form produces alkaline phosphatase, acid phosphatase, leucin arylamidase and naphthol-AS-β1-phosphophdrolase and remains genetically unchanged suggesting that it is highly likely to be viable. It was found that specialized attachment sites such as the "adhesion pedestal", "cup-like indentation" and "abutting adhesion" were seen in the interaction between coccoids and epithelial cells. These adherence patterns were similar to those observed with spiral form in gastric biopsy specimens in vivo, suggesting coccoid could be a differentiated infective form of *H. pylori*[4]. Therefore, this form was suspected to play a critical role in the transmission of *H. pylori* and could be one of the causes of recrudescence of *H. pylori* infection after antibiotic treatment. In this study we investigated mouse immune response against *H. pylori* after oral infection with the bacterium and demonstrated coexistence of spiral and coccoid forms of *H. pylori* in mouse.

MATERIALS AND METHODS

Animals

Female BALB/c mice weighing about 25g were obtained from the Laboratory Animal Center, National University of Singapore. Mice were 5 weeks old when they were sent to laboratory and maintained for one week to allow them to adapt to the new environment. Mice were fed with a

commercial rodent diet and provided with sterile water.

Bacterial strain

An isolate of *H. pylori* H132 obtained from a patient with gastric cancer was used for this study. Strain H132 was isolated on chocolate blood agar base No.2 medium with 5% horse blood at 6 days of incubation of biopsy at 37℃ under microaerophilic environment. The bacterium was inoculated into brain heart infusion (BHI) broth supplemented with 10% horse serum and 0.4% yeast extract in a flask at 37℃ for 2 days. The brother culture was centrifuged at 4000×g for 20min. The supernatant was discarded and fresh BHI broth supplemented with 10% horse serum and 0.4% yeast extract was added to the pellet. The suspension was mixed gently. The inoculum was incubated at 37℃ for another 2 days. The concentration of spiral form was determined by spread plate method and bacterial counting chamber. In this experiment the concentration of *H. pylori* spiral form was about 1-5×10^8CFU/ml.

Animal experimental design

Fifty mice were included in this experiment. They were divided into eight groups. Two groups with ten mice each. One of these 2 groups served as negative control without any inoculation while the second group of 10 mice was inoculated with 0.3ml of 5 m $NaHCO_3$ and 0.3mL BHI serving as internal negative control. The remaining 30 mice were divided into six groups of 5 mice each. Mice in each experimental group were first inoculated with 0.3ml 0.5M $NaHCO_3$. An hour following that, 0.3ml of *H. pylori* suspension was administered with a gastric gavage. The procedure was repected 3 times at 2-day interval for these 30 mice.

Two mice from the controls and five mice from one infection group were sacrificed at weekly interval postinfection. Before being sacrificed, the mice were fasted for one day with free access to water. The mice were sacrificed by cervical dislocation. Stomachs were dissected for microbiological analyses. Five hundreds microliters of blood samples were taken from the heart of sacrificed mice for immune response studies.

Microbiological analyses

Gastric samples were examined within one hour. Samples of antrum were trimmed and inoculated on chocolate blood agars with antibiotics (vancomycin 6g/L, nalidixic acid 5 g/L, amphotericin 6 g/L and trimethoprin 10g/L) and without antibiotics. Plates were incubated in microaerophilic atmosphere at 37℃ for 14 days. Typical colonies were indentified by standard methods[5]. Blood of mice was collected from heart and centrifuged at 4000×g for ten minutes. Sera were removed from clot and stored at -20℃. Sera were examined for immunoglobulins against *H. pylori* by ELISA.

ELISA

Antigens of spiral and coccoid form of *H. pylori* were prepared by acid glycine extraction according to a modification method of Goodwin *et al*[6] as described by Vijayakumari *et al*[4]. Protein concentration was determined by the modified Lowry protein assay and the antigens were stored in 1ml aliquots at -20℃ until use.

The stock antigen solution was diluted in carbonate buffer (90% 0.5M Na_2CO_3 and 10% 0.5M $NaHCO_3$, pH 9.6). Aliquot of 200μL of the diluted antigen preparation was added to each well of a microtitre plate (Nunc) to give 1μg of antigen per well. Plates were left for 24 hours at 4℃. Excess antigen was removed and each were was replaced by 300μl of serum diluent (0.02% timerosal, 0.05% Tween 20 and 1g/L gelatin in PBS) and kept at 4℃ for at least 24 hours before used.

The sera to be tested were diluted 1:100 with serum diluent (0.02% thimerosal, 0.05% Tween 20 and 1g/L gelatin in PBS). A 100μl aliquot of each diluted test serum was added to each of the three wells of the microtitre plate. Plates were incubated at room temperature for 90 minutes. The plates were then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20.

The second antibody was horse radish peroxidas labelled goat anti-mouse immunoglobulins (Dako) which react with all mouse IgG subclasses, IgA and IgM. It was diluted to 1:4000 with PBs containing 0.02% thimerosal, 0.02% BSA and 0.1% gelatin. A 100μl of the diluted secondary antibody was added to each well of the plate that was subsequently incubated at room temperature for another 90 minutes. It was then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20 followed by washing two times with PBS containing 0.02% thimerosal only. A 100μl of substrate containing 40mg of o-phenylenediamine dihydrochloride (OPD, Sigma) and 40μl of 30% hydrogen peroxide (Merck) in 100ml phosphate citrate buffer (0.1M $C_6H_8O_7H_2O$ and 0.2M $Na_2HPO_4H_2O$) at pH 5.0 was added to each well. Plate was left at room temperature in the dark for 15 minutes. A-50μl of 2.5M H_2SO_4 was added to each well to stop reaction. The optical density (OD) of the reaction mixture was read immediately at wavelength of 490nm and 620nm reference filter using an ELISA

reader (Ceres 900 Bio-Ted).

RESULTS

After inoculation *H. pylori* was isolated in only one mouse from one week postinfection. The isolate was identified by spiral morphology, Gram negative, urease positive and naphthol-AS-B1-phosphohydrolase, leucine arylamidase and alkaline and acid phosphatase in API ZYM test. No *H. pylori* was detected in control mice and mice inoculated with $NaHCO_3$ and BHI. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with $NaHCO_3$ and BHI and 77% (23/30) mice infected with *H. pylori*.

In macroscopic findings, no visible gastric erosion or ulceration was seen in either *H. pylori*-infected mice or control mice.

The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The distribution of OD values and the mean OD values of antibodies against *H. pylori* spiral antigens in different groups were shown in Figure 1. The sera of negative control group or the group inoculated with BHI gave very low absorbance except one mouse in negative control group with an OD of 0.616. The mice showed immune responses to *H. pylori* spiral antigens one week after infection with *H. pylori*. Two weeks postinfection, the mean OD value was doubled than that of negative control group (P<0.05). The peak mean absorbance was four weeks postinfection which showed six times higher than that of negative control group (P<0.01). However, mouse serum antibodies against *H. pylori* spiral antigens decreased gradually 5 weeks postinfection.

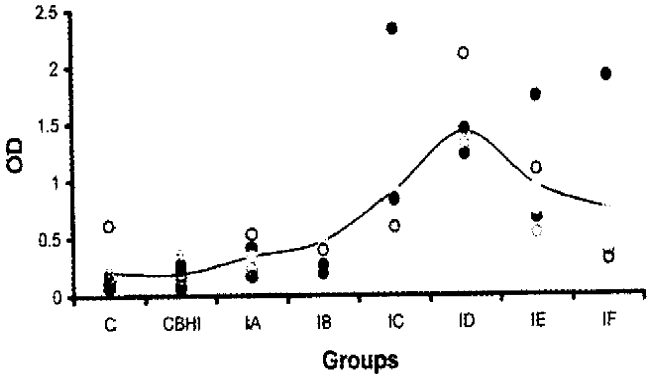

Figure 1 Distribution of Mouse antibodies against *H. pylori* spiral antigens.

C: negative control. CBHI: mice inoculated with $NaHCO_3$ and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five week postifinfection. IF: six weeks postinfection. The line represents mean OD value.

The mouse antibodies against *H. pylori* coccoid antigens were also detected. The distribution of OD values and the mean OD values of mouse serum antibodies against *H. pylori* coccoid antigens in different groups were shown in Figure 2. One week postinfection, the antibodies against coccoid antigens could be detected (P < 0.05) and were almost 4 times higher than that of control. Four weeks postinfection, mouse serum antibodies against *H. pylori* coccoid antigens in infection group were about 18 times higher than that of control group (P <0.01). The mouse serum antibodies against *H. pylori* coccoid antigens decreased gradually 5 weeks postinfection.

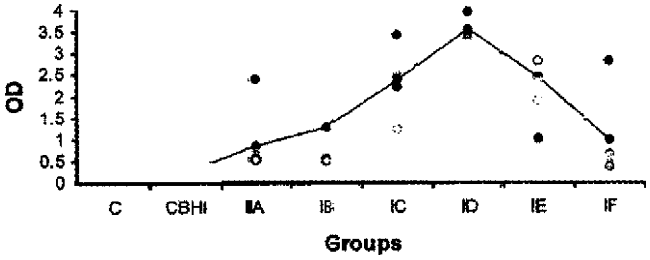

Figure 2 Distribution of OD of mouse antibodies against *H. pylori* coccoid antigens.

C: negative control. CBHI: mice inoculated with $NaHCO_3$ and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five weeks postinfection. IF: six weeks postinfection. The line represents mean OD value.

DISCUSSION

Anumber of animal models have been developed to provide information on pathogenesis, immunity and therapy for *H. pylori* infection. These include models in nonhuman primates[7,8], gontobiotic and conventional piglets[9,10]. However, there were contradictory reports in murine model study. Karita et al[11] detected colonization of *H. pylori* in the germfree athymic and euthymic mice up to 10 weeks after inoculation, but observed temporary colonization in conventional euthymic mice. Cantorna and Balish[12] tried in vain to colonize clinical strains of *H. pylori* in the alimentary tract of germfree rodents. Marchetti *et al*[13] were able to detect colonization of bacterium and gastric pathology in specific-pathogen-free mice up to 8 weeks following challenge with fresh clinical isolates whereas a laboratory strain failed to establish infection. Watanabe *et al*[14] successfully demonstrated the long-term infection with *H. pylori* induces adenocarcinoma in Mongolian

gerbils.

In the present study, attempts were made to colonized specific-pathogen-free mice with a fresh *H. pylori* isolate. *H. pylori* was isolated in one mouse. Culture of this fastidious micro-organism is always a challenge with regard to the proper conditions and interfering contaminants which may inhibit the growth of *H. pylori.* This may be the reason that in this study only one *H. pylori* strain was isolated from one mouse. Urease test was reported to be a highly effective detection method especially in the absence of other microflora as in human stomach or germfree animals[15,16]. However, this method of detection could be ineffective when used in conventional mice due to the presence of other urease-producing microflora which could lead to false positive results[17]. In this study, urease test was found positive in the gastric specimens of 50% (5/10) control mice, 70% (7/10) mice inoculated with $NaHCO_3$ and BHI and 77% (23/30) mice infected with *H. pylori*. The indiscrimination of urease test results among different experimental groups of mice made it difficult to determine whether positive urease tests were caused by *H. pylori* colonization or contaminating urease producers. The result is in agreement with report of Xia *et al*[17] that urease test may not be a suitable method of detection in conventional or specific-pathogen-free mice model.

The serum immune response of mice against *H. pylori* spiral and coccoid antigens was investigated. One week postinfection, the mouse antibodies against *H. pylori* increased. The peak mean OD values of antibodies were four weeks postinfection. Coincidently, the profile of antibodies against coccoid antigens was similar. It was shown that four weeks postinfection, antibodies against coccoid antigens increased much higher than that of spiral antigens. Could there be more coccoid form than spiral form in the stomach of mice where the environment is not so favourable to the growth of *H. pylori*? Bhatia *et al*[18] observed the effect of the presence of Lactobacillus acidophilus or its metabolites on inhibition of *H. pylori* growth in *in vitro* culture. The presence of significant number of *Lactobacillus* in gastrointestinal tract of mice might affect *H. pylori* and promote it to convert to coccoid form. The detection of antibodies against *H. pylori* spiral and coccoid antigens is consistent with the observation in patients with gastroduodenal disease that both forms coexist in stomach and could involve in the outcomes of different gastric disorders[19]. It was reported that after antibiotic treatment for *H. pylori* infection, if failure happened the majority of patients were recrudescence[20], i.e. the patients re-infected with same strains of *H. pylori.* This relapse occurred between 5-50 months after treatment, while 4 weeks after treatment those patients showed eradication of *H. pylori* based on micribiological methods. One reason could be that two forms of *H. pylori* coexist in stomach. When the environmental condition is unfavourable to *H. pylori*, most of the cells might convert to coccoid form which is difficult to be detected by commonly used microbiological methods.

In this study we demonstrated coexistence of spiral and coccoid forms of *H. pylori* in experimental mice. This factor should be considered in clinical management since coccoid form might be viable and pathogenic as suggested by some investigators[3,4].

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A cross-sectional study on HGV infection in a rural population *

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Subject headings GB virus-C; hepatitis G virus; non-A, non-B hepatitis; hepatitis B virus; hepatitis C virus; enzyme-linked immunoassy; polymerase chain reaction

Abstract

AIM To determine the epidemiological characteristics and clinical significance of HGV infection, and to compare with HBV and HCV infections.

METHODS Anti-HGV, HBsAg, anti-HBs, anti-HBc and anti-HCV were detected by enzyme-linked immunoassays (EIA). Anti-HGV positive sera were further tested for HGV RNA by a nested reverse transcription polymerase chain reaction (RT-nPCR).

RESULTS The anti-HGV prevalence rate was 12.9% in the rural population. It was relatively low in children under 10 years of age, and then increased with age and peaked in the group of 50-59 years (29.2%). The Carrier rate of HBsAg was 12.6% in the population and quickly reached the highest (16.2%) in the 5-year age group. The prevalence rate of HBV infection was 64.9%, and rose to a high level in the group of 10 years, and maintained high till up to the top of 79.2% in the 50-59 age group. The HCV infection rate was 15.3%. No Anti-HCV positive cases were found in the group under 10 years of age. It was particularly high in the 20-40 age group, and reached the peak in the group of 30 years old. No significant differences were found in the infection rates of HBV, HCV and HGV between male and female. HGV infection was associated with the history of blood donation and the sexual transmission. The anti-HGV positive rate in wives of husbands with HGV infection was 53.3%, significantly higher than that in those with anti-HGV negative husbands (7.8%). HGV coinfection with HBV or HCV had no influence on serum alanine aminotransferase (ALT). No ALT elevation was found in the group with HGV infection alone.

CONCLUSION The epidemiological characteristics of HGV infection are different from that of HBV and HCV. HGV is transmitted by blood and sex, and does not seem to cause liver damage.

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Received 1998-08-02

INTRODUCTION

Since the discovery of hepatitis C virus (HCV) in 1989. 90% of blood-borne non-A, non-B hepatitis cases, acute as well as chronic, are attributed to HCV. The remaining 10%-15% patients with non-A, non-B hepatitis have no evidence of HCV infection, indicating the existence of additional causative agents. GBV-C and HGV were newly discovered putative non-A to E hepatitis viruses reported by two groups of investigators[1,2]. However, the sequence homology analysis of the two viruses revealed that they are different isolates of the same virus and tentatively designated GBV-C/HGV. It is a positive single-stranded RNA virus, and has a similar genome organization as the flaviviruses, in particular, hepatitis C virus (HCV), and is classified in the same genus[3]. GBV-C/HGV is transmitted mainly through blood or blood products. This study was carried out in a rural population with a high proportion of plasma donors in Zhoukou Area, Henan Province of China, to determine the epidemiological characteristics and clinical significance of HGV infection, and to compare with that of HBV and HCV infection.

MATERIALS AND METHODS

Subjects

All 541 registered residents in the village of Zhoukou Area, Henan Province were investigated.

Data collection

Every resident enrolled in this study received a

questionnaire including 17 items such as age, sex, hepatitis history, blood donation history, etc., and all questionnaires were filled out by the investigators. Then 3.5ml blood was drawn from each resident and the serum was immediately separated, and stored at -20℃ until tested.

Laboratory tests

All residents were tested for serum alanine aminotransferase (ALT) levels by Reitman's method; antibody to hepatitis G virus (anti-HGV), hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis C virus (anti-HCV) by enzyme-linked immunoassays (EIA); and HGV RNA by RT-nPCR. The anti-HGV EIA kit was developed by our laboratory[4]; HBsAg, anti-HBs, anti-HBc and anti-HCV EIA kits were produced by Shanghai Ke Hua Co.. RT-nPCR kit for detection of HGV RNA was established by our laboratory[5].

Diagnostic Criteria

The diagnosis of HGV infection was made on the reactivity of anti-HGV in serum. HBV infection was diagnosed by the presence of one of HBV markers (HBVM) including HBsAg, anti-HBc and anti-HBs. Individuals previously inoculated with HBV vaccines were excluded from the study. HCV infection was diagnosed on the basis of anti-HCV positivity in serum.

Statistical analysis

Frequency distributions and dichotomous variables were performed using the two-tailed Mantel-Haenszel chi-square test or the two-tailed Fisher's exact test (EPI-INFO software). Logistic regression analysis (SPSS statistical package) was applied to identify the independent variables associated with HGV, HBV or HCV infection. *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Age and sex distribution of HGV, HBV and HCV infections

The anti-HGV positive rate was 12.9% in the rural population. Fourty-two of 70 anti-HGV positive individuals tested were also HGV RNA positive (60%). The anti-HGV prevalence rate was relatively low in children under 10 years, and then increased with age and peaked in the group of 50-59 years (29.2%). The HBsAg carrier rate was 12.6% in the population, and quickly reached the highest (16.2%) in the 5-year old group. The prevalence rate of HBV infection was 64.9% in the population. It increased to a high level in the group of 10 years of age, and maintained high up to 79.2% in the 50-59 age group. The anti-HCV positive rate was 15.3% in the population. No anti-HCV positive cases were found in the group under 10 years of age. The anti-HGV prevalence was particularly high in the 20-40 age group, and reached the peak in the group of 30 years. It was 2.2%, 28.2%, 40% and 32.7% in the age groups of 10, 20, 30 and 40 years, respectively, and decreased quickly in the group above 50 years (Table 1).

No significant differences of HBV, HCV and HGV infection rates were found between male and female.

Table 1 Age distribution of HGV, HBV and HCV infection in the rural population

Age group	Cases	Anti-HGV(+)		HBsAg(+)		HBVM*(+)		Anti-HCV(+)	
(yrs)	tested	No.	%	No.	%	No.	%	No.	%
0-	52	1	1.9	7	13.5	25	48.1	0	0.0
5-	74	3	4.1	12	16.2	42	56.8	0	0.0
10-	92	10	10.9	13	14.1	62	67.4	2	2.2
20-	110	12	10.9	12	10.9	68	61.8	31	28.2
30-	70	12	17.1	10	14.3	47	67.1	28	40.0
40-	55	9	16.4	6	10.9	39	70.9	18	32.7
50-	48	14	29.2	10.4	5	38	79.2	2	4.2
60-	40	9	22.5	3	7.5	30	75.0	2	5.0
Total	541	70	12.9	68	12.6	351	64.9	83	15.3

* One of HBsAg, anti-HBc and anti-HBs positive

Epidemiological factors of HGV, HBV and HCV infections

Among 17 doubtful factors tested by single factor analysis, the blood donation history, anti-HBs, anti-HBc and HBVM (one of HBsAg, anti-HBs and anti-HBc) were related to HGV infection. The hepatitis history and age were risk factors for HBV infection, while the blood donation history, ALT level and HBsAg were associated with HCV infection. Multifactors were further analyzed using non-conditional logistic regression. Table 2 shows the risk factors correlated with HGV, HBV and HCV infections.

Table 2 Non-condition logistic regression analysis of HGV, HBV and HCV infections

Markers	Related factors	B value	OR value	*P* value
Anti-HGV	Blood donation history	0.6759	1.97	<0.05
	Anti-HBc	0.7629	2.14	<0.05
HBsAg	Hepatitis history	1.1079	3.03	<0.05
	Blood donation history	-1.0001	0.37	<0.05
	Anti-HBs	-1.8481	0.16	<0.001
	Anti-HBc	2.3166	10.14	<0.001
HBVM	Hepatitis history	0.9554	2.59	<0.05
Anti-HCV	Blood donation history	5.0103	149.95	<0.001
	Frequency of plasma donation	2.6594	14.29	<0.05
	HBsAg	-2.7363	0.06	<0.01
	ALT level	1.1172	3.06	<0.05

Anti-HGV positive rate was not correlated to the duration and frequency of plasma donation, whereas anti-HCV positive rate was associated with them. The anti-HCV positive rate of individuals with plasma donation more than 1 year (32/37, 86.5%) was significantly higher than that of those with less than 1 year (45/69, 69.2%) or without plasma donation (6/433, 1.4%).

Analyses of HGV, HBV and HCV infection between couples

Eighty-three couples were divided into two groups: in one group, both wife and husband had blood donation, and in another group, only one or neither of the couple had blood donations. The results showed that anti-HGV and HBVM positive rates in wives of husbands with anti-HGV or HBVM were significantly higher than in wives of anti-HGV or HBVM negative husbands ($P<0.001$ and $P<0.05$, respectively). However, no significant correlation was found in HCV infection between wives and husbands (Table 3).

Relationship between ALT and HGV, HBV and HCV infections

The abnormal rate of ALT in the individuals with HCV infection (34.5%) was significantly higher than in those with HGV or HBV infection (0% and 6.6%).

Table 3 Positive rates of anti-HGV, HBsAg, HBVM and anti-HCV of wives and husbands

Husbands infection	Wives anti-HGV positive rate(%)		Wives HBsAg (%)		Wives HBVM (%)		Wives anti-HCV (%)	
status	A	B	A	B	A	B	A	B
+	40.0 (2/5)	53.3 (8/15)	0 (0/1)	11.1 (1/9)	60.0 (6/10)	72.0 (36/50)	68.8 (11/16)	11.1 (1/9)
-	8.3 (1/12)	7.8 (4/51)	17.6 (3/17)	8.9 (5/56)	62.5 (5/8)	40 (6/15)	100.0 (4/4)	7.4 (4/54)
Total	17.6 (3/17)	18.2 (12/66)	16.7 (3/18)	9.2 (6/65)	61.1 (11/18)	64.6 (42/65)	75.0 (15/20)	7.9 (5/63)
OR value	7.4	13.4	0	1.3	0.9	3.9		0
χ^2	2.29	16.1	0.20	0.04	0.01	5.09	1.58	0.14
P value	<0.05	<0.001	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05

*A: Both had blood donations; B: One or neither had blood donations

Table 4 Relationship between ALT and HGV, HBV and HCV infections

Group	No. tested	ALT abnormal rate (%)	*P* value
HBV-HCV-HGV+	11	0.0(0/11)	>0.05
HBV-HCV-HGV-	145	5.5(8/145)	
HBV+HCV-HGV+	46	8.7 (4/46)	>0.05
HBV+HCV-HGV-	256	6.6(17/256)	
HBV-HCV+HGV+	5	60.0(3/5)	>0.05
HBV-HCV+HGV-	29	34.5(10/29)	
HBV+HCV+HGV+	8	50.0 (4/8)	>0.05
HBV+HCV+HGV-	41	43.9(18/41)	

DISCUSSION

The enzyme-linked immunoassay (EIA) for detection of anti-HGV used in this study was established by our laboratory. The total coincidence rate between HGV RNA RT-nPCR and anti-HGV EIA kits was 94%, as reported previously[4]. So the anti-HGV positive rate determined by anti-HGV EIA reflects the actual status of HGV infection in the population. The epidemiological characteristics and risk factors of HGV infection in the population appear to be different from that of HBV and HCV.

The anti-HGV positive rate was 12.9% in the

population, significantly higher than that in the general population of China[6]. The HBsAg carrier rate and the prevalence of HBVM in this rural population were 12.6% and 64.9%, respectively, which were similar to that of the general population in China reported by Liu *et al*[7]. However, the anti-HCV positive rate of this population (15.3%) was much higher as compared with the general population of the country (15.3% *vs* 3.2%)[8]. The high prevalence of HGV and HCV infections may be associated with the high proportion (19.6%) of plasma donors in the rural population.

The age distributions of HGV, HBV and HCV infections were different. The anti-HGV positive rate was relatively low in children under 10 years, and then increased with age and peaked in the group of 50-59 years (29.2%). However, the HBsAg carrier rate quickly reached the highest (16.2%) in the 5-year age group, and the prevalence rate of HBVM increased to a high level in the group of 10 years of age, and maintained high up to 79.2% in the 50-59 age group. HCV infection in the population had a special pattern of age distribution different from HGV and HBV. It mainly concentrated in groups of 20, 30 and 40 years of age, with the prevalence rates of 28.2%, 40.0% and 32.7%, respectively. No anti-HCV positive cases were found in the groups under 10 years of age. The high-prevalence rate of HCV infection in the groups of 20-40 years was related to the high proportion of plasma donors among them.

The anti-HGV positive rate in wives of husbands with HGV infection was 53.3%, significantly higher than that in those with HGV negative husbands (53.3% *vs* 7.8%).The same phenomenon is also seen in HBV infection. The prevalence rate of HBVM in wives of husbands with HBV infection was significantly higher as compared with those of husbands without HBVM (72% *vs* 40%). Although the anti-HCV positive rate in Wives of husbands with HCV infection was relatively higher than that in those of anti-HCV negative husbands (11.1% *vs* 7.4%), but there was no statistical significance. The data demonstrated that the sexual transmission of HGV and HBV seems to be more important as compared with HCV.

The ALT abnormal rate in the individuals with HCV infection alone was significantly higher than that in those with HGV or HBV infection alone (34.5% vs 0% or 5.6%). It is interesting to note that the ALT levels in HBV patients with or without HGV coinfection had no difference[6]. It suggests that HGV, unlike HBV and HCV, may not cause the liver damage[2,9,10].

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Persistence of hepatitis B vaccine immune protection and response to hepatitis B booster immunization *

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Subject headings hepatitis B vaccines; immune protection persistence; booster; immunization

Abstract

AIM To identify the persistence of immune protection of China-made, plasma-derived hepatitis B vaccine after infancy immunization and the time table of booster immunization.

METHODS A cross-sectional follow-up study and an experimental study on booster were used for the evaluation of the serological effect 7 years after vaccination and the antibody anamnestic response. Radioimmunoassay was used for the detection of hepatitis B virus markers.

RESULTS The protective anti-HBs positive rates of 1018 children, who were vaccinated according to the regimen of three doses of 10 μg hepatitis B vaccine in their infancy, declined from 75.0% during the first two years to 48.2% in the 7th year after the first dosage, however, the positive rates for HBsAg and anti-HBc always fluctuated at a low frequency. A total of 144 subjects aged 6 or 7 years, who were negative for both HBsAg and anti-HBc before booster, were selected from 1018 children of the follow-up study, and boosted with 1μg intradermally or 2μg hypodermically hepatitis B vaccines. Their anti-HBs GMT and anti-HBs positive rates were 190.6mIU/ml and 89.6% in the first month after booster, significantly higher than 14.7mIU/ml and 54.9% before booster ($P < 0.01$), and declined back to 25.3mIU/ml and 75.5% in the 12th month; among 65 children with the anti-HBs negative before booster, 40 had a level of anti-HBS ⩾100mIU/ml one month after booster, suggesting retention of immune memory in most of them.

CONCLUSION No need for revaccination against hepatitis B in the 7th year after the initial immunization due to better persistence of immune protection of the vaccine and retention of immune memory to hepatitis B virus in the vast majority of the vaccinees.

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Received 1998-11-09

INTRODUCTION

Infant hepatitis B vaccine immunization integrated with EPI program has become a principal strategy for the hepatitis B control. Since the end of the 1980s, large-scale hepatitis B vaccination in infants has been implemented in the many areas of China[1]. The short-term effectiveness of hepatitis B vaccine has been confirmed in many studies[1-4]. The low-dose immunization has been recommended as a principal strategy to infancy vaccination of the rural areas[5]. However, it is necessary to answer the following questions in community-based hepatitis B prevention: what is the persistency of immune protection of China-made, plasmaderived hepatitis B vaccine, especially in the infancy should low-dose immunization be used? Is there antibody anamnestic reaction to hepatitis B surface antigen (HBsAg) in the vaccinees with vaccine-induced antibody negative-conversion When should the booster immunization be administered? In order to determine the duration of immune protection and the immune memory to HBsAg 7 years after the infancy vaccination, a follow-up study on the long-term effectiveness of hepatitis B vaccination and an experiment study of hepatitis B booster immunization were carried out in Longan County, a remote hepatitis B endemic rural area of China, from 1994 to 1995.

MATERIALS AND METHODS

Sample size and subjects

A total of 1018 children aged 1-7 years, born in the period of 1987 to 1994 in Longan County and

having the vaccination record of three doses of 10μg plasma-derived hepatitis B vaccine (produced by the National Institute for Biological Products, Beijing) according to 0, 1 and 6 month schedule, were selected in terms of cluster sampling as a sample for the observation of long-term effectiveness. Among them 144 children were recruited as subjects for the experiment of booster immunization.

Method and dosage of booster

The 144 subjects were divided into two groups. One group of 91 subjects were intradermally injected with a dose of 1μg plasma-derived hepatitis B vaccine, another group of 53 subjects were hypodermically immunized with a dose of 2μg vaccine.

Reagents

Hepatitis B radioimmunoassay (RIA) reagent kits were purchased from the National Institute of Biological Products, Beijing.

Specimens collection and lab test

Peripheral blood of 3ml-5ml was collected in all samples in April 1994, and from all subjects at 1- and 12 month after the booster, respectively. Serum specimens were kept at -20℃ for the test. RIA was used for the detection of anti-HBs, anti-HBc and HBsAg. Anti-HBs-S/N ratio ≥10.0, anti-HBc inhabitation ratio ≥75% and HBsAg S/N ratio ≥ 2.1 were defined as sera positive. Both scales, anti-HBs mIU/ml GMT and anti-HBs positive rate, were used for comparison of the differences of antibody level between before and after booster, and between different doses of booster vaccine. The following formula was used for calculating anti-HBs mIU/ml:

$$\text{mIU/ml} = 130.75\ [\ \text{EXP}\ (\ 0.66765 \times \frac{\text{CPM of sample-CPM of negative control}}{\text{CPM of positive control-CPM of negative control}}\ ) -1]$$

Data analysis

Softwares, dBase-III and SAS, were used for the data base and the statistical analysis.

RESULTS

Positive rates for anti-HBs, anti-HBc and HBsAg 1-7 years after immunization

The age distribution of positive rates for anti-HBs, anti-HBc and HBsAg of 1018 immunized children aged 1-7 years after infancy hepatitis B vaccination is shown in Table 1.

Table 1 shows that the anti-HBs positive rate significantly declined from 75.0% of age group of 1-2 years to 48.2% of 7-year age group (X^2=51.2, P<0.01), while anti-HBc and HBsAg positive rates were not found significantly increased with age (P >0.05). The results suggested that the hepatitis B vaccine induced-antibody level in infancy immunization was decreasing year by year after vaccination, however, the difference of hepatitis B virus (HBV) infectious rate between age groups was not statistically significance.

Change of anti-HBs before and after hepatitis B vaccine booster

The results of comparison of anti-HBs level change of 144 subjects before and in the first and 12th month after booster are shown in Table 2.

Anti-HBs GMT of 144 subjects one month after booster was significantly higher (by 18.3 fold) than that before booster (t = 17.4, P < 0.01). However, in the 12th month after booster, the anti-HBs GMT of 106 subjects dropped significantly, and there was no difference before and after booster (t =1.3, P> 0.05); and the anti-HBs positive rates were 89.6% in the 1st month and 75.5% in the 12th month, significantly higher than (54.9%) before booster (P<0.05). The antibody positive rates of both subgroups with low anti-HBs titer (10mIU/ml-99mIU/ml) and the subjects with negative anti-HBs (<10.0mIU/ml) in the first month after booster were significantly lower than before (P<0.01), increasingly recovering in the 12th month.

Relationship of anti-HBs level before and after booster

Anti-HBs level distribution after booster among the subjects with different antibody level before booster is shown in Table 3.

Table 1 Positive rates for anti-HBs, anti-HBc and HBsAg of 1018 children aged 1-7 years after infancy hepatitis B immunization in Longan County in 1994

Age group (yr)	No. of subjects	Anti-HBs(≥10S/N)		Anti-HBc(≥75%)		HBsAg(≥2.1S/N)	
		n	%	*n*	%	*n*	%
1-2	220	165	75.0	1	0.5	2	0.9
3-4	341	178	52.2	13	3.8	9	2.6
5-6	320	144	45.0	8	2.5	7	2.2
7	137	66	48.2	8	5.8	1	0.7
Total	1018	553	54.3	30	3.0	19	1.9

Table 2 Comparison of anti-HBs level before and after hepatitis B booster in 144 subjects immunized with hepatitis B vaccine

Time point of observation	No. of subjects	Anti-HBs (mIU / ml)								GMT	*t*	*P*
		<10		⩾10		⩾100		⩾1000				
		n	%	*n*	%	*n*	%	*n*	%			
Before booster	144	65	45.1	58	40.3	20	13.9	1	0.7	10.4		
One month after booster	144	15	10.4	16	11.1	100	69.4	13	9.0	190.6	17.4	<0.01
12 months after booster	106	26	24.5	43	40.6	33	31.1	4	3.8	25.3	1.3	>0.05

*blood specimens only collected from 106 children in the 12th month after booster.

Table 3 Distribution of anti-HBs levels one and twelve months after booster among immunized children with different anti-HBs levels before booster

Anti-HBs (mIU / ml) before booster	Anti-HBs levels (mIU / ml) one month after booster									Anti-HBs levels (mIU / ml) 12 months after booster								
	No.	<10		⩾10		⩾100		⩾1000		No.	<10		⩾10		⩾100		⩾1000	
		n	%	*n*	%	*n*	%	*n*	%		*n*	%	*n*	%	*n*	%	*n*	%
<10	65	15	23.1	10	15.4	35	53.9	5	7.7	47	22	46.8	14	29.8	9	19.1	2	4.3
⩾10	58	0	0.0	5	8.6	49	84.5	4	6.9	45	4	8.9	24	53.4	15	33.3	2	4.4
⩾100	20	0	0.0	1	5.0	15	75.0	4	20.0	13	0	0.0	5	38.5	8	61.5	0	0.0
⩾1000	1	0	0.0	0	0.0	1	100.0	0	0.0	0	0	0.0	0	0.0	1	100.0	0	0.0

One month after booster 76.9% of 65 subjects with negative anti[a2]HBs before booster, had anti-HBs ⩾10mIU/ml; 91.4% of 58 subjects with anti-HBs low titer possessed anti-HBs ⩾100mIU/ml; in 20 of 21 subjects with anti-HBS ⩾100mIU/ml antibody increased obviously and decreased in one. Fifteen of 65subjects with anti-HBs <10mIU/ml before booster, were still anti-HBs negative in the first and 12th month after booster, accounting for 23.1%, in 10 children anti-HBs level increased from 10mIU/ml to 100mIU/ml and in the remaining 40 subjects it was 100mIU/ml or over. Thirty-two individuals of the latter two groups were followed up for 12 months, 7 had antibody negative conversion.

Tables 2 and 3 indicate that most of immunized children possessed immune memory to HBsAg, and also in some individuals who had the vaccine-induced anti-HBs negative-conversion, 6 or 7 years after their infancy immunization.

Comparison of anti-HBs level between two booster dosages with different immunization routes

Anti-HBs GMTs of 91 subjects of intradermal 1μg group and 53 subjects of hypodermical 2 μg group before booster were 9.3 mIU/ml and 11.3mIU/ml, and no statistically significant difference was found between the two groups (t = 0.6, P>0.05). One month after booster, anti-HBs GMTs of both groups increased to 160.8mIU/ml and 247.2 mIU/ml. However, there was no statistical difference between both groups (t = 1.3, P > 0.05). The difference of booster-induced antibody levels between the intradermal group and the hypodermical group was not found in this study.

DISCUSSION

The observation should be conducted from two aspects to study the persistence of hepatitis B immunization: ①the trend of change of anti-HBs, anti-HBc and HBsAg after vaccination in the given immunized populations; ②to clarify whether the immunized individuals possess immune memory in the certain period after vaccination. The evidence of their immunological anamnestic reaction to HBsAg can be provided through a booster experiment.

The results of our study in Longan County showed that the anti-HBs positive rate of the immunized children was 48.2% in the 7th year, lower than 75.0% during the first two years after infancy hepatitis B immunization, suggesting that the hepatitis B vaccine-induced protective antibody level was gradually decreasing; however, the positive rates for anti-HBc and HBsAg were not significantly increased with the time after vaccination, but obviously lowered than before immunization. The following two explanations might be used for this phenomenon: ① an assumption that the opportunity exposed to HBV was obviously decreased in the immunized population. In recent years the large-scale infant hepatitis B vaccination did significantly decrease the HBsAg carrier rate among the children aged under 5 years[2-4], while, the HBsAg carrier rate in the

older-age population did not decrease, shown in our another study on the population aged 20-30 years without hepatitis B vaccination in Longan County in 1995. ② A part of immunizxed population were found to have vaccine-induced anti-HBs negative-conversion, but they might still have immune memory to HBsAg. If these children expose to HBV, they will quickly develop enough protective antibody to avoid becoming a HBsAg carrier. The second explanation has been confirmed through a hepatitis B vaccine booster experiment in our study.

The results of the booster experiment indicated that 61.5% (40/65) of 65 subjects with anti-HBs, anti-HBc and HBsAg negative marker, yielded anti-HBs level of ⩾100mIU/ml one month after a low dose of hepatitis B vaccine booster, and the post-booster antibody increase of these children was referred to immunological anamnestic reaction, according to the standard that the anamnestic reaction was defined as subjects with the anti-HBs-negative yielding anti-HBs level of ⩾100mIU/ml four weeks after booster[6]. Therefore, the reason why the HBsAg positive rate of immunized population always fluctuated at a low level of around 2% was probably attributable to the fact that they still keep immune memory 6-7 years after the initial vaccination. In 50 of 65 subjects with the anti-HBs and HBsAg-negative the antibody increased obviously after booster. It is interesting that 21.9% (7/23) of those children with anamnestic reaction had antibody negative-conversion in the 12th month after booster, and 23.1% (15/65) of subjects were anti-HBs negative at in the first and 12th month after and before booster. The outcome when these two groups of children expose to HBV should be observed in the future. Of 79 subjects with antibody level of ⩾ 10mIU/ml, 68 (86.1%) had antibody level increased by 2-fold or more one month after booster, suggesting that a low dose of hepatitis B vaccine booster can induce extremely high titer of antibody in most of these children.

The results of our research are similar to that of a study on booster 4-5 years after infancy hepatitis B vaccination by Chen Hui-Fang[7]. Both studies reveal that the majority of children immunized with China-made, plasma-derived hepatitis B vaccine, can quickly produce antibody anamnestic reaction to HBV (titer ⩾100 mIU/ml) 4-7 years after infancy.

These evidences indicate that low dose of China-made, plasma-derived hepatitis B vaccine in infancy may yield a better persistency of immunization and an ideal protective effect in immunized population. It is suggested that no need for revaccination against hepatitis B in the 7th year after the initial immunization, due to no evidences of booster obtained in our study.

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Effect of lipid on proliferation and activation of rat hepatic stellate cells (I)

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Subject headings Hepatic stellate cell; triglyceride; very low-density lipoprotein; cell proliferation

Abstract

AIM To study the effect of lipid (triglyceride and very low-density lipoprotein, VLDL) on proliferation and activation of rat hepatic stellate cells (HSC).

METHODS HSC were isolated and cultured from liver of Wistar rats by in situperfusion with pronase and collagenase and density gradient centrifugation with Nycodenz. HSC proliferation was examined with MTT colorimetric assay.

RESULT Triglyceride of 12.5 mg/L had a promoting effect on proliferation of HSC ($P<0.05$), 25, 50, 100 and 200 mg/L had no effects ($P>0.05$), but 400 mg/L had an inhibiting effect ($P<0.01$). VLDL of 6.25 and 12.5 mg/L had no effect on proliferation of HSC ($P>0.05$), but increased concentration of VLDL could promote the HSC proliferation ($P<0.05$).

CONCLUSION Lipid had an effect on proliferation of HSC. Triglyceride and VLDL may promote HSC proliferation and may be associated with fatty liver and hepatic fibrogenesis.

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Received 1998-09-07

INTRODUCTION

Despite the early controversy over the primary cellular source of extracellular matrix proteins in liver fibrosis, compelling *in vitro* and *in vivo* experimental evidence is now available to implicate activated hepatic stellate cells (HSC) in the pathogenetic role[1-3]. Mechanisms by which collagen-producing cells are activated under pathological conditions remain unknown and continue to be a topic of research interest[1,2,4,5]. Although the role of hepatocytes in lipid metabolism and transportation have been discussed intensively, that of HSC was not known More and more studies showed that HSC took part in lipid metabolism and transportation[6], their abnormality was related to the pathogenesis of fatty liver and liver fibrosis[7]. In order to seek a possible explanation for the role of lipid in activation of HSC in liver fibrogenesis, we observed the effects of triglyceride and very low-density lipoprotein (VLDL) on proliferation of rat HSC.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 400g-450g were fed ad libitum with standard rodent chow.

Preparaton of HSC

HSCs were prepared by the methods of Friedman *et al*[8] and Baroni *et al*[9] with slight modifications. The rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg). The liver was perfused in situ through the portal vein with 500ml of calcium-free Gey's balanced salt solution for 10min at a flow rate of 40ml/min-50ml/min and then enzymatically digested with perfusate containing 0.05% collgenase (Sigma) and 0.1% pronase E (Merck) for 10min-15min. After being removed, the liver was cut into small pieces and incubated in 50ml fresh GBSS-BSA containing 0.05% collagenase and 0.1% pronase E and stirred at 37℃ for 30min. After passing through gauze, cell suspension was centrifuged at 500×g for 7 min, the supernatant discarded at the cells washed twice further with Dulbeco's modified Eagle's medium (DMEM) (Gibco). The cell suspension was mixed with 18% (W/V) of Nycodenz (Sigma) in GBSS without NaCl. The gradient was centrifuged at 1450

×g for 17min at 4℃. The white, diffuse, flufly band in the lower region of the DMEM layer just above the Nycodenz cushion, which contained highly enriched HSC, was gently aspirated, diluted in about 30ml DMEM and centrifuged at 450×g for 7min at 4℃. The cell pellet was suspended in the incubation medium and seeded in the culture flasks with DMEM containing 20% fetal calf serum. Sterile condition was maintained during the entire isolation and purification procedures. Cell viability was assessed by trypan blue exclusion, and cell counting were conducted in a hemocytometer.

Culture and determintion of HSC[8,9]

Purified HSC suspended in DMEM containing HEPES (15mmol), penicillin (100U/ml), streptomycin (100 mg/ml) and fetal calf serum (20% V/V) were seeded at a density of 1×10^5 cells/cm^2 in the culture flasks. The medium was changed 20 to 24 hours after plating, and every 3 to 4 days thereafter. Cells were grown at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. To evaluate the purity of the cultures, HSC at day 2 or 3 and 7 after plating were tested by immunohistochemistry staining for desmin, lysozyme and factor VIII-related antigen, and by ultraviolet excited fluorescence microscopy at the length of 328 nm.

Proliferation of HSC

HSC proliferation was studied by colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma) assay[10]. HSC (1×10^5cells/100 μl) were cultured in multiwell tissue culture plates (96 well/plate) (Corning, New York, NY, USA) for 2 days and 48hrs in the presence or absence of triglyceride and VLDL. Twenty μl of MTT solution (5mg/L) was added to all wells of an assay, and plates were incubated at 37℃ for 4hrs. Then 100μl dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 20min-30min at room temperature to ensure that all crystals were dissolved, the plates were read on a E-Liza Mat-300 reader, using a test wavelength of 570nm, a reference wavelength of 630nm. Plates were normally read within 1hr of adding the dimethyl sulfoxide. All samples were analyzed in pentaplicate.

Statistical analysis

All data were expressed as mean ±SD. Statistical differences were assessed by the standard *t* test and *P* values of <0.05 were judged to be statistically significant.

RESULTS

The yeild, viability and purity of HSC

The yield of HSC ranged from 5×10^6 to 1×10^7 cells per liver. HSC displayed fading green-blue fluorescence with fluorescence microscopy at a wave length of 328nm. After the first washing at 24hr, the HSC purity in culture exceeded by 95% as assessed by phase contrast microscopy and ultraviolet excited fluorescence microscopy. Viability of HSC assessed by trypan blue exclusion exceeded by 95%. HSC positive for desmin by immunohistochemistry in primary culture was above 90%, and in subculture above 95%. Lysozyme and factor VIII-related antigen were negative for HSC.

Effect of triglyceride on proliferation of rat HSC

Trigylceride was directly administered to HSC at concentrations of 12.5, 25, 50, 100, 200 and 400mg/L, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation in the presence of triglyceride is shown in Table 1. Compared with the contrast (0.1395± 0.0276), 12.5 mg/L of triglyceride had a promoting effect on proliferation of HSC ($P<0.05$), 25, 50, 100 and 200mg/L had no effects ($P>0.05$), but 400mg/L had an inhibiting effect ($P<0.01$).

Table 1 The effects of triglyceride on HSC proliferation

Group	Concentration (mg/L)	Value of OD
Triglyceride	400	0.0990±0.0163[b]
	200	0.1226±0.0138
	100	0.1212±0.0275
	50	0.1450±0.0264
	25	0.1637±0.0243
	12.5	0.1894±0.0316[a]
Normal control		0.1395±0.0276

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

Effect of VLDL on proliferation of rat HSC

VLDL was directly administered to HSC at concentrations of 6.25, 12.5, 25, 50 and 100 mg/L of VLDL, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation is shown in Table 2. Compared with the contrast (0.1395 ± 0.0276), 6.25 and 12.5mg/L of VLDL had no effect on proliferation of HSC ($P>0.05$), but 50 and 100mg/L of VLDL could promote HSC proliferation ($P<0.05$ or $P<0.01$).

Table 2 The effects of VLDL on HSC proliferation

Group	Concentration (mg/L)	Value of OD
VLDL	100	0.2202±0.0284[b]
	50	0.1964±0.0287[b]
	25	0.1834±0.0498[a]
	12.5	0.1642±0.0269
	6.25	0.1583±0.0314
Normal control		0.1395±0.0276

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

DISCUSSION

There is now overwhelming evidence that HSC, which reside in the space of Disse, are the principal effectors of hepatic fibrogenesis. However, in contrast to most other conditions, it is far from clear whether the characteristic phenotypic transformation, proliferation and fibrogenesis by HSC is always a response to liver cell injury/ or inflammation.

HSCs were demonstrated to present lipoprotein receptor in their surface, contained a large amount of fat droplets in their cytoplasm, and synthesized and secreted apolipoproteins. HSCs were in direct contact with plasma in the Disse's space, accessable to both chylomicron and VLDL triglyceride, and do not need to transport the enzyme to the adjacent endothelium. All these suggested that HSC and hepatocytes were necessary for lipid mobilization and transportation[1,2]. Their abnormality was related to the disturbance of lipid mobilization and transportation which made triglyceride concentrate in the liver, inducing fatty liver and liver fibrosis[12]. Reeves *et al*'s study[7] showed that the mechanism of HSC activation/ proliferation and subsequent fibrogenesis related to alcohol may be unrelated to necroinflammation, and that HSC activation occurced in the absence of hepatitis in alcoholic liver disease and correlated with the severity of steatosis. However, isolated fatty liver without hepatitis occurs more frequently than does steatohepatitis. Thus it is not clear whether the accumulation of fat in the liver is responsible for inflammation or whether inflammation evoked by some stimulus caused cell dysfunction that resulted in steatosis[12]. Vicente *et al*[11] studied the lipid metabolism during in vitro induction of the lipocyte phenotype in HSC. HSC can produce and bind lipoprotein lipase to their own surface, using it potentially for processing exogenous lipids in the acute phase of lipocyte induction, when the accumulation of lipids was accelerated. In the our study, we found that triglyceride and VLDL promoted HSC proliferation at certain concentrations. Some animal studies also confirmed that steatosis and collagen content in experimental liver cirrhosis are affected by dietary monounsaturared and polyunsaturated fatty acids[12,13]. The great amount of lipid in diet produced fatty liver and liver fibrosis more easily. These experimental evidence indicated that the infiltration of lipid increased the production of fibrous tissue in the liver. The earliest event in the development of fibrosis appears to be activation of HSC by such factors as lipid peroxides[14,15]. This caused proliferation of HSC and initiation of fibrogenic cascade in the liver. In addition, the investigation showed that high concentration of triglyceride (400mg/L) had an inhibiting effect on HSC proliferation. It was deduced that high concentration of triglyceride might have a toxic effect on HSC. The precise role of triglyceride and VLDL in the HSC proliferation and activation needs to be addressed in futher studies.

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In situ hybridization assay of androgen receptor gene in hepatocarcinogenesis *

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Subject headings hepatocarcinoma; androgen receptor; *in situ* hybridization; liver neoplasm, experimental

Abstract

AIM To determine the correlation between expression of androgen receptor (AR) gene and hepatocarcinogenesis.

METHODS Male SD rats were used as experimental animals and the animal model of experimental hepatocarcinoma was established by means of 3′-me-DAB administration. Androgen receptor mRNA was detected by a non-radioactive *in situ* hybridization assay in neoplastic and non-neoplastic liver tissues.

RESULTS The expression of androgen receptor mRNA was observed only in neoplasticcells and some atypical hyperplastic cells. In the liver tissue of control animal and the remaining normal liver cells adjacent to the carcinoma tissue, no positive signal was seen.

CONCLUSION Androgen has an important correlation with hepatocarcinogenesis and the expression of androgen receptor gene might be a mark event during hepatocarcinogenesis.

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***Supported by the National Natural Science Foundation of China, No. 39570348**

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Received 1998-08-21

INTRODUCTION

It is well known that sex hormone plays an important role in regulation of cell growth, organ development and carcinogenesis. But the most majority of studies were focused on the tumors of sex hormone-dependent organs (e.g., mammary carcinoma and prostate carcinoma). By now not much attention has been paid to sex hormone effects on hepatocarcinogenesis. However, some data showed that there is a difference in morbidity and mortality of patients with hepatocarcinoma between male and female[1,2]. Hepatic adenomas have been also demonstrated to have a clear relationship with oral contraceptive use, and it was presumed that there may be hormone receptors within the adenoma cells that mediate tumor growth in response to hormonal stimulation[3]. In addition, some reported that testosterone stimulated tumor growth and it may enhance the progression of chemically-induced hyperplastic nodules to frank malignancy[4]. All these suggested that hepatocarcinoma might also be a hormone-dependent tumor. In this study, male SD rate were used as experimental animals and the animal model of experimental hepatocarcinoma was established by means of 3'-me-DAB administration. Androgen receptor mRNA was detected by a non-radioactive *in situ* hybridization assay in neoplastic and non-neoplastic liver tissues in an attempt to detect the inner link between the expression of androgen receptor gene and hepatocarci nogenesis, and explore its exact mechanism.

MATERIALS AND METHODS

Establishment of animal model of experimental hepatocarcinoma

Male SD rats were divided into two groups: ① control group: the animals were fed with standard food; ② experimental group: the animal were fed with the food containing 0.6% 3'-me-DAB, after 14 weeks the food were replaced with standard one. The experimental rats were sacrificed at 4, 8, 14, 17 and 24 weeks respectively. The liver tissue was fixed in 10% formaldehyde and embedded in paraffin.

Preparations of RNA probe

A fragment of androgen receptor cDNA was cloned in a transcription vector Bluescript between *Eco*RI

and *Hind* III. By means of transcription *in vitro*, a RNA probe complementary to the rat AR mRNA was labeled with digoxigenin. The probe was stored at -20℃.

In situ hybridization

The sections, that represent different stages of hepatocarcinogenesis, were selected for *in situ* hybridization assay. After deparaffin and rehydrate, the sections were fixed in 4% paraformaldhyde again. The hybridization was carried out at 50℃ and placed overnight. The hybrids were then revealed by an alkaline phosphatase-conjugated anti-digoxigenin antibody and detected with the detection system of Boehringer Mannheim.

RESULTS

Pathological changes in different stages of hepatocarcinogenesis

SD rats were divided into 10 groups as shown in Table 1. The specimen from various groups were first used for pathological examination. Results in different stages of experiment has demonstrated a gradual progression of hepatocarcinogenesis. At the 4th and 8th week, hyperplastic foci and nodules appeared, and the carcinoma nodules were seen at the 24th week of experiment.

Table 1 Groups of experimental animals

	4 weeks	8 weeks	14 weeks	17 weeks	24 weeks
Experimental group	5	5	5	5	5
Control group	3	3	3	3	3

Expression of AR gene in different stage of hepatocarcinogenesis

The selected sections from different groups, which reflect the pathological characteristics of hepatocarcinogenesis in different stages, were used for *in situ* hybridization assay. The rsults showed that no positive signal of AR expression was seen in the early stage of hepatocarcinogenesis (the 4th and 8th week). The signal of AR expression could be detected until the 14th week of experiment (Table 2), and all the hybridization signal was observed in the carcinoma tissues. In the liver tissues of control animal, and the remaining normal liver cells adjacent to the carcinoma tissue, no positive signal was detected (Figure 1A, B). In the experimental group of the 14th and 17th week, some atypical hyperplastic cells also displayed positive signal in varying degrees (Figure 1C, D). In all of control group, no positive signal was seen.

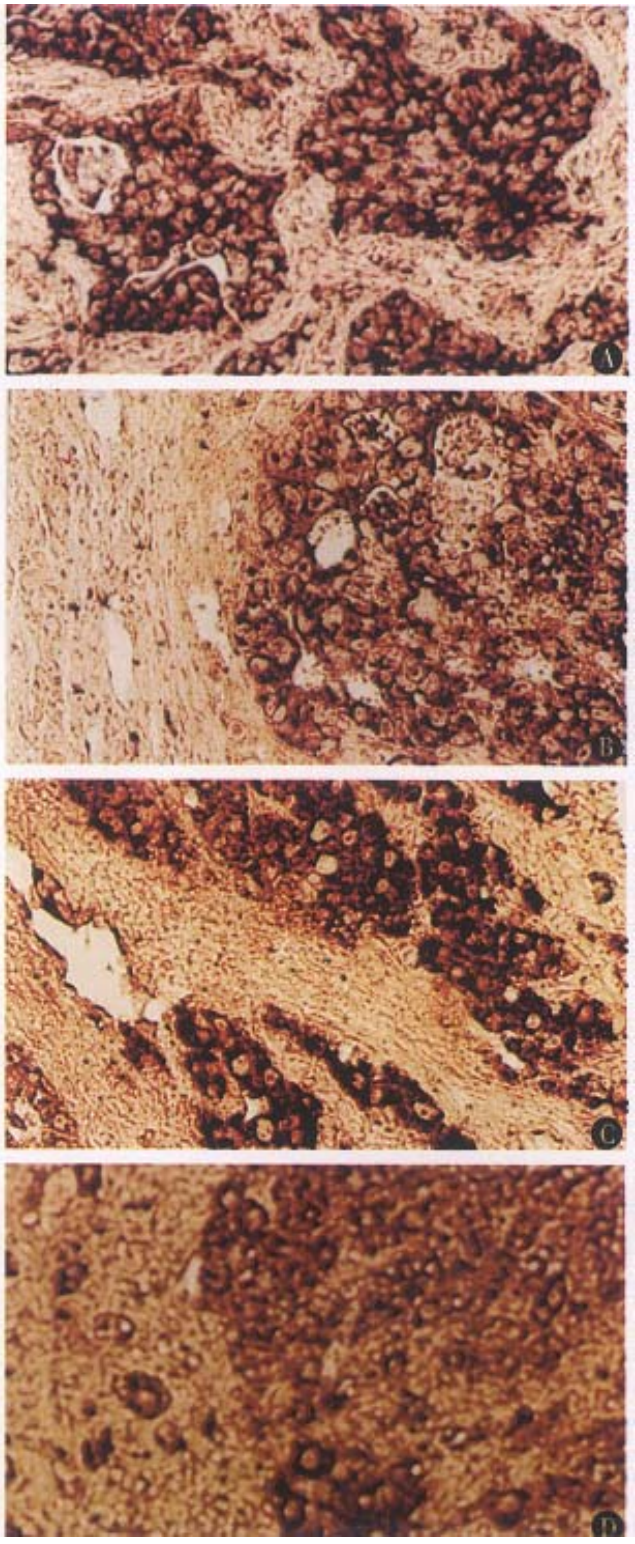

Figure 1 Expression of androgen receptor mRNA in hepatocarcinogenesis (*in situ* hybridization).

A: Liver from the 17th week experimental group, shows the expression of AR in the carcinoma tissue.

B: Liver from the 24th week experimental group, shows the expression of AR in the carcinoma tissue and in the remaining normal tissue adjacent to the carcinoma tissue.

C: Liver from the 14th week experimental group, shows the expression of AR in the atypical hyperplastic cells.

D: Liver from the 17th week experimental group, shows the expression of AR in the atypical hyperplastic cells.

Table 2 Expression of AR in deferent stages of hepatocarcinogenesis

	4 weeks	8 weeks	14 weeks	17 weeks	24 weeks
Experimental group	-	-	+	+	+
Control group	-	-	-	-	-

DISCUSSION

It is not clear yet whether androgen plays a role in regulation of hepatocarcinogenesis. But many data showed that there might be a correlation between androgen and hepatocarcinogenesis. The regional data in *Cancer incidence in five continents* demonstrated that the morbidity in men is generally higher than in women, no matter where the incidence is high, moderate or low[1]. Besides some men¡äs unhealthy hobby (e.g., excessive drinking) and work surroundings, the potential effects of sex hormone is also a factor which can not be ignored. Another data revealed that the danger of contracting hepatocellular adenoma increased among the women who use oral contraceptives[5]. Some also reported that the patients, who use the steroid-hormone stimulating metabolism of androgen over a long period of time for aplastic anaemia therapy, are susccptible to liver cancer. It is also observed that in some patients the survival was prolonged and the tumors were reduced in size after stopping the hormone-therapy[6]. In view of these data, it has been presumed that there might be some relationship between androgen and hepatocarcinogenesis. However, this conjecture was only based on the clinical observation and the statistical data, it lacks strong experimental proof yet. Our experimental results showed that the expression of androgen receptor mRNA was observed in the atypical hyperplastic cells and in the cells of hepatocellular carcinoma (the 14th, 17th and 24th week), yet in the normal liver tissue and the tissue from the early stage of hepatocarcinogenesis (the 4th and 8th week) no positive signal was seen. This result prompted us further that androgen probably issomewhat related to hepatocarcinogenesis. The strong expression of androgen receptor mRNA in hepatocarcinoma cells and no expression or weak expression (the level of expression might be lower than the threshold for detection) in the normal liver tissue and the remaining normal liver cells adjacent to the carcinoma tissue showed that androgen had an important bearing on hepatocarcinogenesis. It should be noted that androgen receptors express also in varying degrees in the atypical hyperplastic cells. Because the atypical hyperplsia is considered as a precancerous stage, the expression of androgen receptor gene in this stage give us much for thought. Is the synchronism of the appearance of precancerous cells and the expression of androgen receptor a mere coincidence or a necessity? This question is well worth further studying. According to these results, we infer further that androgen has an important correlation with hepatocarcinogenesis and the expression of androgen receptor gene might be a mark event during hepatocarcinogenesis.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):506-510

Sequencing of *p*53 mutation in established human hepatocellular carcinoma cell line of HHC4 and HHC15 in nude mice

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Subject headings Liver neoplasms; carcinoma, hepatocellular; *p*53 gene; mutation; HHC4; HHC15; Tumor cell, cultured

Abstract

AIM To set up cell lines of human hepatocellular carcinoma in nude mice for the research of cell biology and gene therapy.

METHODS Xenotransplantation of human hepatoma into nude mice was carried out and the growth rate, histopathology and immunology of the nude mice were studied. The DNA from xenografts were analyzed by HBV gen and PCR amplification of a fragment of *p*53 gene exon 7, which were identified by dot blot hybridization, restriction fragments length polymorphism and DNA sequencing.

RESULTS HHC4 and hHCC415 cell lines could be successively transplanted in nude mice and the population doubling time was 7 and 5 days respectively. These strains retained the original characteristics of histopathology, secreting AFP and heteroploid karyotypes in human hepatocellular carcinoma. The fragment of HBV gene was detected in the genomic DNA of both hHCC4 and hHCC15, however only hHCC4 secreted HBsAg. The mutation at 250 code (C→A) and 249 code (G→T) were detected respectively in the genomic DNA of HHC4 and HHC15.

CONCLUSION The two cell lines are useful material for the studying of cell biology and gene therapy in human hepatocellular carcinoma and provide molecular biological trace of the relationship between high mortality of hepatoma and AFB1 severe pollution of the daily common foods in this district.

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***Supported by the Science Foundation of Department of Publish Health of Fujian, No.**85003-01-11

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Received 1998-11-09

INTRODUCTION

Although the etiopathology of human hepatocellular carcinoma (hHCC) is still unknown, a lot of evidence strongly suggested that infection of HBV and contamination of aflatoxin B_1 (AFB_1) were the inducing fuctos of the carcinogenesis of hHCC. Xenografts of human tumorous tissue in nude mice usually retain their original morphology, antigen, karyotype and function. These models can be used for several purposes, including assessment of the etiopathology, cell biology, sensitivity of chemotherapy, genetherapy, and so on. hHCC4 and hHCC15 cell lines were established from the patients of HHC in Tong'an district of Xiamen where there was high adjust death rates of hHCC (44.75 per one hundred thousand population from 1987 to 1989) which was the secondary mortality of hHCC in China. In this district, a lot of evidence has been shown that there was severe contamination of AFB1 in daily common foods, such as the oil of peanut (92.9%) and fermented soy beans (44.4%)[1]. Also high epidemic infection of HBV was presented in the population in this country (17.6%)[2]. Because the relationship between molecular biological changes and etiopathology of HHC in this district is still absent hHCC4 and hHCC15 cell lines in nude mice were studied by cell biology and molecular biology.

MATERIAL AND METHODS

Animals

Male and female nude mice, about 4 to 6 weeks old, with BALB/C genetic background were provided by Medical Experimental Animal Laboratory, Cancer Research Center, Xiamen University, where the mice were bred and maintained in vinyl box isolated under specific pathogene free condition. The sterilized food pellet and tap water were given ad libitum.

Xenotransplantation

Tumor tissues of patients, who underwent partly hepatotomy in the Min-Hai Hospital, Tong'an, were dissected from the primary site in the liver and aseptically minced and placed in cooled culture medium. Several tumor tissue fragments about 2 mm in diameter were transplanted, with trocar, into the subcutaneous tissue of the back of 6 mice within 3

hours after surgical removal of the tumor. Afterwards, a piece of tumor tissue from hHCC15 was orthotopically transplanted into the liver of 21 nude mice for studying the ability of secreting AFP from tumor.

Growth

Tumors in the subcutaneous tissue or liver were measured in 3 dimensions with calipers for every 7 days. Tumor size was plotted on a graph.

Morphology

For light microscopy and transition electron microscopy (TEM) the primary tumors of liver and xenografts were fixed and stained by the standard methods.

Assay of AFP, HBsAg and HBcAg

The Sera from patients and tumor bearing mice were analyzed radioimmunologically with AFP Diagnosis Kit (The Institute of Biochemical Assay and Product, Shanghai). In brief, 100μL of blood was obtained weekly for 6 weeks, by orbital venipuncturing from mice. Two cell lines were inoculated subcutaneously or into liver (for the later only with hHCC15) respectively. HBsAg and HBcAg in the sear of tumor-bearing mice were analyzed with HBsAg and HBcAg Kit (New and Advanced Co. Ltd Xiamen) by ELISA method.

Analysis of chromosome

According to the standard method of preparing metaphase cell treated with colchicine, chromosome in hHCC4 and hHCC15 cell line was carried out. The number of chromosomes in each spread cell was counted and all of spread cells were done at least for 100.

Amplification of HBV DNA fragment

The DNA from xenografts were extracted with the standard method of Sambrook and assayed by the HBV Diagnosis Kit (a kind gift from professor Wu Bing-Qun, Dept. of Pathology Beijing Medical University, China). Forward and reverse primer for HBV fragment were designed as follow: F 5'-GGGTGGAGCCCTCAGGCTCAGGGCA-3', R 5'-GAAGATGAGGCATAGCAGCAGGAT-3'. The positive and negative samples were amplified simultaneously as control. The products of PCR were run in 12g/L agarose gel stained with ethidimum bromine and photographed.

Amplification of p53 gene fragment

DNA of two strains was a mplified by PCR to produce target of a 110 bp at seventh exon of *p*53 gene using primers of P1 5'-GTTGGCTCTGACTGTACCAC-3' and P2 5'-CTGGAGTCTTCCAGTGTGAT-3' on DNA Thermal Cycler 480 (Perkin-Elmer/Cetus). The product of 110bp DNA fragment was identified by 20g/L agarose gel electrophoresis and DNA dot blot hybridization which was performed with hDIG-labeled p53 cDNA probe (2.0kb, cut from reconstructed plasmid ph *p*53β, a kind gift from Professor Liu Si-Li, Tianjin Medical College). The probe was labeled as the described method of DIG DNA Labeling and Detection Kit (Boehringer Mannhemi).

Analysis of restriction fragments length polymorphism (RFLP)

Five to 10μl of above PCR amplified products were digested with 7 to 10 unit Hae III restriction enzyme at 37℃ for 6hr, then precipitated with cooled ethanol. The sediments were analyzed with 150g/L non-denatural polyacrylamide gel electrophoresis, and then stained with ethidium bromide and visualized under UV light.

Sequencing of PCR products

The 110bp of PCR amplification fragments were purified by standard low-melting point agarose gel electrophoresis method and labeled with fluorescence according to the description of Tag Dye Deoxy™ Terminator Cycle Sequencing Kit. The DNA sequence of PCR fragments were analyzed and edited by Applied Biosystems 373A DNA Sequencer.

RESULTS

Transplantation and growth

The neoplasams of hHCC4 and hHCC15 were presented in the back of 1 in 6 and all 6 mice respectively and showed rapid growth after several generations. A 58% and 100% rate of tumor transplantation were presented in hHCC4 and in hHCC15 respectively. Xenografts were shown a short latency (18.7 days ±4.9 days and 17.5 days ±1.6 days respectively) and almost stable after successive generations. The population doubling time in hHCC4 and hHCC15 cell line was about 7 and 5 days respectively, the growth curves are shown in Figure 7.

Morphology

Most of the transplanted tumors retained approximately the original morphological characteristics (Figure 1). No metastasis foci was presented in the liver and lung of each tumor-bearing mice within 6 weeks. Ultrastructurally, sinusoid like structure and bile canaliculi were scattered between two cells (Figure 2). Under TEM, fibrillary structure of HBsAg could be seen in the rough endoplasmic reticulum (RER) of carcinoma cells in hHCC4 (Figure 3).

Products of AFP, HBsAg and HBcAg

Radioimmunoassay disclosed human AFP in sera of both groups mice bearing xenografts of both

strains, (until as large as 100mm^3), but not for the control group, and their values increased progressively in relation to orthotopic growth of the tumor in hHCC15 (Chart 2). HBsAg could be detected in hHCC4, but not for HBcAg. HBcAg and HBsAg could not be undetected in hHCC15 by ELISA immunoassay.

Karyology

All evaluable chromosomes of metaphase in hHCC4 and hHCC15 were human chromosomes, and no any mouse chromosomes were seen (Figure 4). A histogram of chromosome counts of 100 cells disclosed the number of chromosomes ranging between 50 to 175, the median number of 106 to 126 in hHCC15 (Figure 9) and 110 to 134 in hHCC4 (data not shown) cell line respectively.

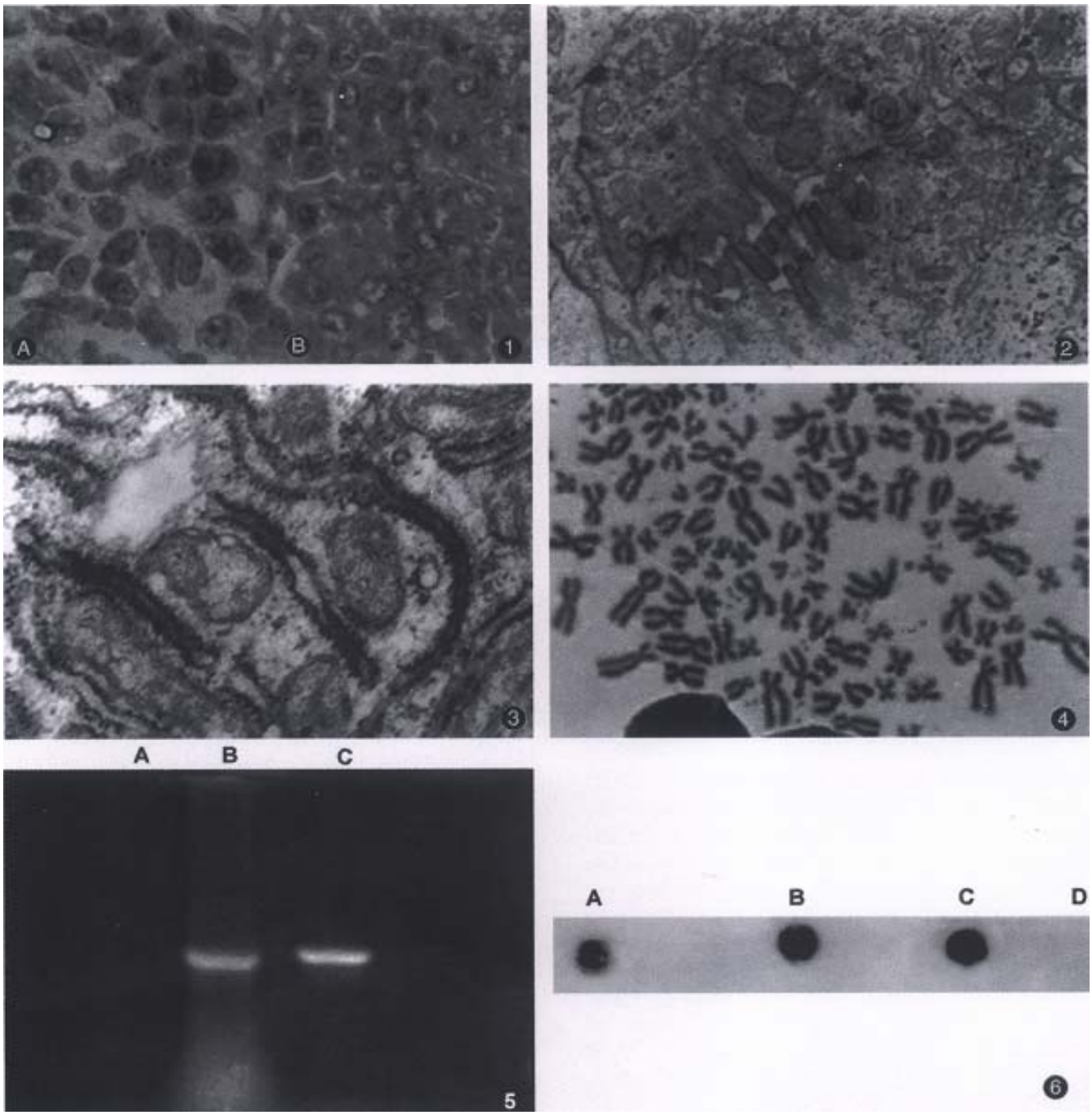

Figure 1 Light microscope of hHCC4 and hHCC15 showing high nuclear-cytoplasmic radio. A: HHC4, B: HHC15
Figure 2 The bile canaliculi between two carcinoma cells. ×15000
Figure 3 The filament of HBsAg in the rough Endoplasmic reticulum of hepatoma cell of hHCC4. ×40000
Figure 4 Karyotype of hHCC15.
Figure 5 Electrophoresis of HBV PCR product from hHCC15. A. negative control, B. HHC15, C. positive control
Figure 6 Dot blot hybridization of *p*53 PCR product. A. hHCC4, B. hHCC15, C. ph *p*53β plasmid for positive control, D. pBR322 for negative control

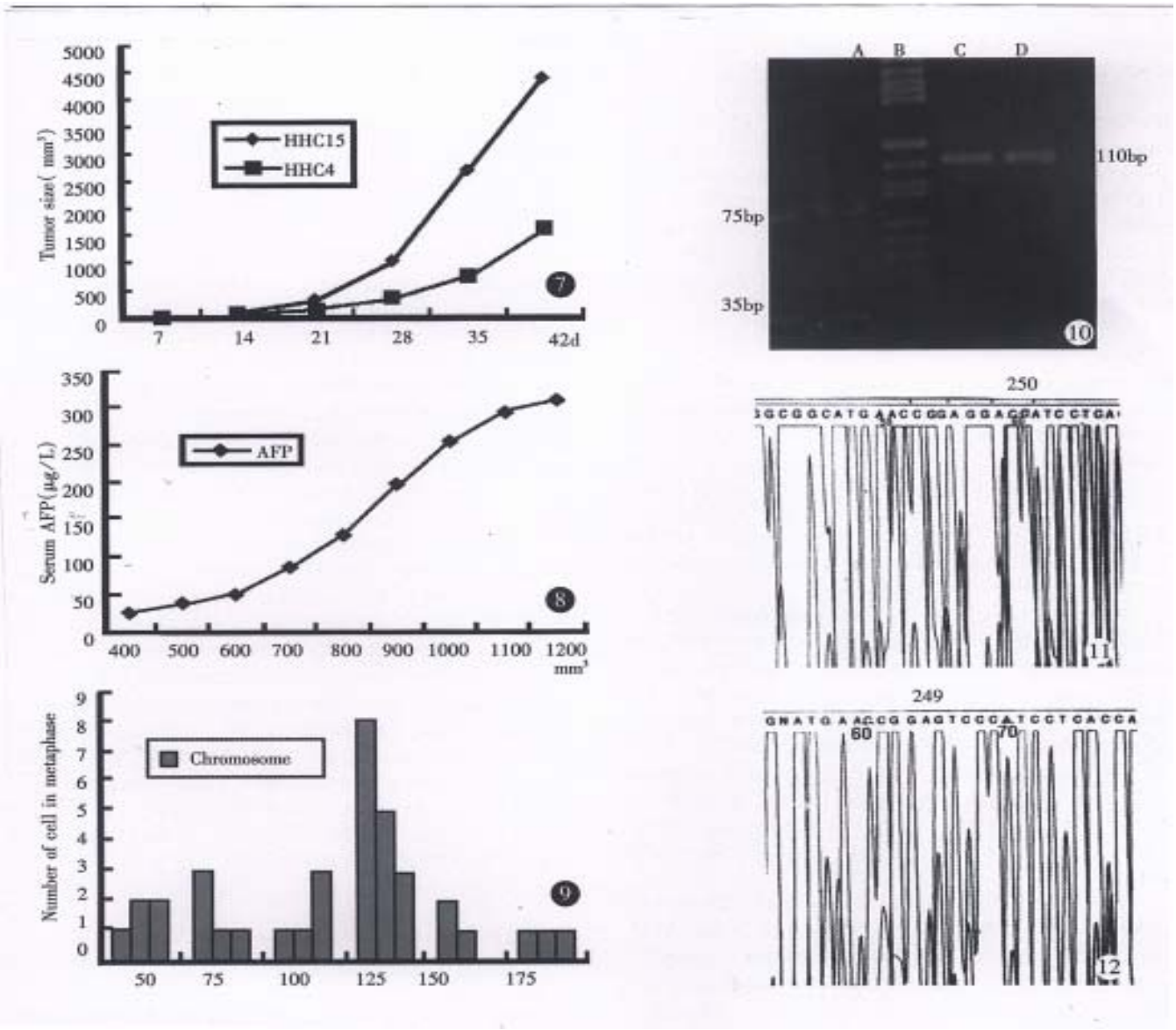

Figure 7 Growth curves of hHCC4 and hHCC15 in nude mice.
Figure 8 The relationship between the volume of orthotopical xenograf and the AFP values in the sera of a nude mouse bearing hHCC15.
Figure 9 The histograms of chromosome numbers in hHCC15 cell passage 21.
Figure 10 Restriction fragment length polymorphism analysis of PCR products. A. ph *p*53β plasmid for positive control, B. DNA marker (pBR322 / Hae III), C. hHCC4, D. hHCC15
Figure 11 Dna sequencing results of partial PCR fragment showing C→A mutation at 250 code of *p*53 gene from hHCC4.
Figure 12 DNA sequencing results of partial PCR fragment showing G→T mutation at 249 code of p53 gene from hHCC15.

Amplification of HBV DNA fragment

PCR products amplified from both hHCC4 (data not shown) and hHCC15 or positive control had similar band running in the same distance in agarose gel, but not any product in negative control sample (Figure 5).

Identification of PCR products and analysis of RFLP

DNA extracted from hHCC4 and hHCC15 xencgrafts and plasmid ph *p*53β containing wild *p*53 cDNA were amplified respectively, then the PCR products were analyzed by DNA dot blot. Figure 6 showed positive hybridization of the PCR products from hHCC4, hHCC15 and ph *p*53β plasmid with the control negative from plasmid pBR322, which meant the good specificity of the PCR system. With the method of RFLP, it was shown that the product of PCR from ph *p*53β plasmid, as a contrast of wild *p*53 cDNA, was digested into two bands of 75bp and 35bp (Line A) and undigested two of 110bp from hHCC4 and hHCC15 (Figure 10). It was suggested that the mutation at seventh exon of *p*53 gene

could be presented in the xenografts of hHCC4 and hHCC15.

Sequencing PCR products

The results showed in Figures 11 and 12 were the partial DNA sequence of fragments amplified from hHCC4 and hHCC15 genomic DNA respectively. Figure 11 revealed a CCC→ACC point mutation in 250 code of *p*53 gene from xenograft of hHCC4, while Figure 12 showed an AGG→AGT mutation of *p*53 gene from xenograft of hHCC15.

DISCUSSION

Xenografts of hHCC in nude mice usually retain their original morphology, antigen, karyotype and function, such as secreting AFP. It has been reported that both AFP and HBsAg can not be detected simultaneously in nude mouse transplanted with hHCC, but are presented in the cell lines of hHCC *in vitro*[3]. The presenting fibrae of HBsAg in the RER of carcinoma cell support the specific function of cells from hHCC4.

A variety of *p*53 mutations have been found in a wide spectrum of sporadic tumors, in which the cause of carcinogenesis was still unknown and direct evidence is absent. It has been reported that mutation of *p*53 gene was the most common inducing factor in primary advanced hHCC. AFB_1 was the most inducing factor of carcinogenesis in hHCC and specially associated with the mutation of code 249 in *p*53 gene, being notable as "hot spots" in hHCC, spreading over South Africa or southeast coast of Asia on the earth[4,5].

Multiple evidence support a closely relationship between AFB_1 and HHC. Rats fed with AFB_1 developed hepatoma in a dose-depend fashion[6]. AFB_1 intake might lead the liver to acute necrosis and proliferation of hepatoid-oval cells[7]. The more AFB1 intake daily was, the more necrosis appeared and the oval cells possessing the ability of division continuously divided.

According to the telomere hypothesis of cellular senescence theory, somatic cells, which continuously divided, lead to cell cycle exit and significant telomere erosion and shortage at which the crisis state (M_1) of cell arrived, which was induced by activating *p*53 and *p*Rb cascade. It is conceivable that *p*53 is proposed to signal a growth cheekpoint allowing cell to arrest in G_0 or G_1 state (replicative senescence) by inducing *p*21 expression which in turn inactivate cdk/cyclin complex leading to underphosphorylation of the Rb proteins[8]. It is reasonable that *p*53 gene mutation caused by AFB_1 have the ability to allow cells to overcome M_1, leading to an extended lifespan until a second growth checkpoint, M_2 is reached. This rare event, M_2, is most often associated with the reactivation of telomerase. During the past few years, there has been mounting evidence that the activation of telomerase, a ribonucleoprotein enzyme, is important in maintaining telomere length stability and necessary for the sustained growth of the most cancer[9]. It has been reported recently that due to reactivating telomerase, mammary epithelial cells, which were transfected with mutation *p*53 gene, could be survive and become immortal *in vitro*[10]. The two cell lines provided molecular biological trace of relationship between high mortality of hepatoma and AFB_1 in this district. It shown that carcinoma cells of hHCC4 and hHCC15 possessing telomerase activity (unpublished data) supported the events of *p*53 mutation discovered by us.

The carcinogenesis of hHCC is closely related to chronic hepatitis B in which the molecular machanism of hHCC is still a mystery. Futhermore, AFB1 treatment of trangenic mice integrated with hepatitis B DNA greatly enhanced the development of hepatoma as compared with the mice not treated with AFB_1[11]. It is wise to use two cell lines for the disclosing the contribution of carcinogenesis of human hepatoma in coordination of HBV integration and *p*53 mutation which were presented in both of the cell lines. Also they are the useful material for cell biology, sensitivity of chemotherapy and gene therapy.

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Segmental transcatheter arterial embolization for primary hepatocellular carcinoma *

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Subject headings liver neoplasms/therapy; carcinoma, hepatocellular/therapy; embolization, therapeutic; portal veins

Abstract

AIM To evaluate the therapeutic effects of segmental transcatheter arterial embolization for primary hepatocellular carcinoma, and to recognize the menifestation and clinical value of lipiodol overflow into portal veins surrounding the tumors.

METHODS A total of 50 cases of nonresectable primary hepatocellular carcinoma underwent segmental transcatheter arterial embolization. Two methods of superselective segmental catheterization were used, one was the method of wire-guiding, and the other the technic of co-axial infusion catheter.

RESULTS The 1-, 2-, 3- and 4-year cumulative survival rates of 50 cases with segmental transcatheter arterial embolization for primary hepatocellular carcinoma were 83.8%, 65.4%, 42.9% and 24.5% respectively. The in cidence of the lipiodol overflow into portal veins was 64%. The overflow of lipiodol intoportal veins, represented as 3-5 grade branches of portal veins visualized by lipiodol, was "star-like" or "tree-like", and there was a relatively large vessel in the center surrounded with radicalized small branches of vessels.

CONCLUSION The lipiodol overflow into portal veins was one of the signs of complete embolization for tumors, and may play a partial role in embolizating the portal venous supply for hepatocellular carcinoma.

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***Supported by "9.5" National Major Project of National Committee of Sciences and Technology, No.**96-907-03-02.

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Received 1998-05-10

INTRODUCTION

Segmental transcatheter arterial embolization (SLp-TAE) has become one of excellent interventional methods for primary hepatocellular carcinoma[1]. It was reported that SLp-TAE may play a dual role in embolizing the hepatic arterial and partly portal venous supply for hepatocellular carcinoma. We have performed SLp-TAE since 1990 in our hospital and accumulated some experience and reported it below.

MATERIALS AND METHODS

Materials

A total of 50 patients (48 men, 2 women) with nonresectable HCC undergoing SegLp-TAE were studied. They ranged in age from 23 to 71 years (mean, 41.8 years ± 4.2 years). The clinical symptoms included pain in upper abdomen, abdominal mass and weight loss. AFP was elevated in 44 patients, and cirrhosis occurred in 35 cases. Child's classification showed 15 cases of Child's A, 33 cases of Child's B, and 2 of Child's C. The main tumor measured 1.5 cm - 10 cm with a mean of 6.0cm±1.4cm (7 cases, >10 cm; 28 cases, 5 cm -10 cm; and 15 cases <5cm). Multiple nodules were found in 10 of the cases.

Methods

Coaxial infusion catheters of 3.0F with 0.013 inch micro-wire (Target Therapeutic Inc.) were used.

The feeding segmental or sub-segmental artery was detected carefully by celiac arteriography, and variants were excluded by superior mesenteric arteriography and phrenic arteriography. Two methods of superselective segment arterial catheterization were employed: one was wire-guiding catheterization, the other was technic of co-axial infusion catheter.

The volume of 2 ml - 20 ml emulsion (AOE, Adriamycin mixed with lipiodol) were injected, and Gelatin sponge particles were used in some patients. Before embolization, 2 ml - 3 ml of 1% lidocraine was injected to prevent vessel spasm.

RESULTS

A total of 80 TAE (mean 1.6) were performed by two methods of segmental catheterization (34 by wire-guiding, 16 by co-axial catheter) with 16 cases of sub-segment, and 34 cases of segment in embolization position. Survival period ranged from 3 to 68 months (mean, 1.9 years ± 1.2 years). The 1-, 2-, 3- and 4-year cumulative survival rates were

83.8%, 65.4%, 42.9% and 24.5% respectively. Complete necrosis was revealed in 3 resected lesions.

DISCUSSION

It is well known that the blood supply of primary hepatocellular carcinoma is mainly from the hepatic artery, but the portal venous supply is important to its growth, especially, in the edge of tumor. Nakamura[2] introduced the segmental transcatheter arterial embolization which may play a dual role in embolizing the hepatic arterial and partly portal venous blood supply for hepatocellular carcinoma, leading to complete necrosis of tumor. The 1- and 2-year survival rates of 50 cases were 83.8% and 62.7% in his reports. In our study, the 1-, 2-, 3- and 4-year cumulative survival rates were 83.8%, 65.4%, 42.9% and 24.5%. The therapeutic results were comparatively good.

Two methods of superselective segmental arterial catheterization were used in our study, one was the wire-guiding catheterization, the other was the technic of co-axial infusion catheter. The successful rate of the former method was 60%-65%. The co-axial catheter was soft, adapted well to the distorted arteries, and could be easily inserted to the segment and sub-segment artery which suppied the tumor. But it is relatively difficult in operation, and need more time of exposure. In our study, we usually inserted the catheter in to proper hepatic artery or lobar artery by the wire-guiding method, before completing the segmental catheterization by the co-axial infusion catheter. The total success rate of segment catheterization was 80%-85%.

In 1988, Nakamura[1] discovered in plain abdominal radiograph immediately after injection of oil emulsion, that part iodized oil would overflow into portal veins surrounding the tumors through arterioportal shunt or communications when a relatively large amount of lipiodol was injected into the hepatic artery. In their cases, there was no arterioportal shunt in hepatic angiography. This phenomenon was confirmed by the animal experiment[3,4]. The occurrence rate of lipiodol overflow into portal veins was 64% (32/50 cases) in this series. It represented 3-5 grade branches of portal veins visualized by lipiodol which were "star-like" and "tree-like", or a relatively large vessel in the center surrounded with small radiate branches.

Some authors[5,6] found in the resected specimen which demonstrated lipiodol overflow into portal veins, that not only complete necrosis of the tumors was achieved, but also partial necrosis or atrophy occurred in the normal tissues near the tumor. It was suggested that overflow of lipiodol into portal veins was one of the marks of complete embolization for tumors, and may play a partial role in embolizing the portal venous supply for the hepatocellular carcinoma.

There were eighteen cases without lipiodol overflow into portal veins in our group, which was probably related to different blood supply for the neoplasm. The more affluence of blood supply, the more opportunity of lipiodol overflow. In our group, 71.9% of the cases demonstrated complete deposit of lipiodol in plain radiograph or CT, and only 2 cases with scarce deposit, which suggested that the more complete deposit of lipiodol, the more opportunity of lipiodol overflow through the arterioportal shunt inside the tumor. Additionally, in our practice the treatment was interrupted sometimes by obvious pain resulting from vessel spasm after a bit lipiodol injection, which probably influenced the occurrence of lipiodol overflow into portal veins.

Because primary hepatocellular carcinoma usually has the properity of multi-center origin, and micro-tumor metastasis to small branches of portal veins, there were multiple foci beyond the tumor-bearing segment at early stage, which may not be detected by conventional CT or angiography. "Two-steps" method of transcatheter arterial embolization was adopted in some cases in which multiple foci may occur. First, segmental embolization was introduced to the main tumor-bearing segment or sub-segment. Secondly, in patient's tolerance, small dose of lipiodol was injected into the whole liver through the proper hepatic artery, so as not to leave out the micro-foci which escape detection before embolization.

CONCLUSION Segmental transcatether arterial embolization has become one of excellent treatments of primary hepatocellular carcinoma[8,9]. The 1-, 2-, 3- and 4-year cumulative survival rates were 83.8%, 65.4%, 42.9% and 24.5% in our group. The long-term prognosis awaits further observations.

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Findings of non-pathologic perfusion defects by CT arterial portography and non pathologic enhancement of CT hepatic arteriography *

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Subject headings liver neoplasms/radiography; carcinoma, hepato cellular/radiography; hepatic arteriography; tomography, X-ray computed

Abstract

AIM To recognize the characteristic findings of non-pathologic perfusion defects with CT arterial portography (CTAP) and nonpathologic enhancement found in CT hepatic arteriography (CTHA).

METHONDS The manifestations of nonpathologic perfusion defects with CTAP and non-pathologic enhancement found in CTHA were analyzed in 50 patients with primary hepatocellular carcinoma.

RESULTS The false-positive rate of perfusion defects detected in CTAP was 15.1%. The shapes of perfusion defects were peripheral wedge, small, round, and patchy. The occurrence rate of non-pathologic enhancement found in CTHA was 22.0%. The shapes of non-pathologic enhancement were small, round, irregular, and wedge.

CONCLUSION There was high frequency of non-pathologic perfusion defects detected with CTAP and non-pathologic enhancement found in CTHA. The simultaneous use of both procedures may help decrease the false-positive rate, and increase the veracity of diagnosis for hepatocellular carcinoma.

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***Supported by "9.5" Natioal Major Project of National Committee of Sciences and Technology, No.** 96-907-03-02

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Received 1998-10-08

INTRODUCTION

CT arterial portography (CTAP) and CT hepatic arteriography (CTHA) are the most sensitive methods of detecting hepatocellular carcinoma[1,2]. In recent years, there are more reports on non-pathologic perfusion defects of CTAP and non-pathologic enhancement CTHA. To better recognize and understand the characteristic manifestation of non-pathologic perfusion defects with CTAP and non-pathologic enhancement in CTHA, we analyzed the CT images of 50 cases of hepatocellular carcinoma on CTAP and CTHA in our hospital from January 1995 to January 1998.

MATERIALS AND METHODS

Materials

Fifty patients (44 man, 6 women) with hepatocellular carcinoma were examined with CTAP and CTHA in our department. They ranged in age from 21 to 65 years (mean age, 413 years). AFP was elevated in 41 patients. Cirrhosis occurred in 42 cases. Child's classification showed 16 cases of Child's A, 30 cases of Child's B, and 4 cases of Child¡äs C. The tumors measured 0.2cm-5.5cm in size with a mean of 3.6cm. Multiple nodules were found in 42 cases.

Methods

CTAP examinations were performed with incremental scanning of liver in cranial-to-caudal direction with 8-mm or 10-mm collimation on bi-spiral Elscint Twin Flash scanner (Elscint Corp.). CT images were obtained 25sec-35sec after the initiation of transcatheter (5-F) superior mesenteric artery injection of 30 ml - 40 ml of non-ionic contrast material. Contrast material was injected at a rate of 3.0ml/sec-3.5ml/sec with an automatic power injector (Medrad, Pittsburgh). Conventional angiography was not performed before CTAP. During the catheterization, contrast material administered before CT scanning was limited to 5ml -10ml injected by hand to visualize any aberrant vessels and to facilitate proper catheter placement.

CTHA was done by injecting contrast material into the proper hepatic artery, the common hepatic

artery, or the celiac artery. The volume of 20ml-30ml of contrast material was injected at a rate of 3.0 ml/sec - 3.5 ml/sec. Consecutive scanning of the liver was started 6sec-8sec after te initiation of injection of contrast material. After CTAP and CTHA examination, 3 ml - 15 ml of lipiodol was injected into the hepatic artery, and plain CT scan of liver was performed after two or three weeks (Lipiodol CT, Lp-CT).

RESULTS

Detectability of tumors

CTAP and CTHA images of 50 cases were interpreted prospectively double-blind by two radiologists, and confirmed pathologically combined with lipiodol deposits in Lp-CT, and 6mo-16mo follow-up (42 tumors of 18 cases proved by operative pathology; 30 lesions of 15 cases by biopsy, and the other 27 cases by Lp-CT and follow-up). A total of 232 tumors were found, including 214 tumors detected by CTAP (the rate of detectability was 92.2%), 209 detected by CTHA (the rate of detectability was 90.1%), and 220 detected by simultaneous use of both procedures (the rate of detectability was 94.8%). Our study did not reveal a statistically significant difference in the sensitivities of CTAP, CTHA and simultaneous use of both procedures.

Non-pathologic perfusion defects of CYAP

A total of 252 perfusion defects were detected with CTAP in the 50 patients, including 38 non-pathologic abnormal perfusion defects in 18 cases with a false positive rate of 15.1%. The shape of perfusion defects was peripheral wedge, small round and patchy. All the non-pathologic perfusion defects were not demonstrated on CTHA images.

Non-pathologic enhancement of CTHA

A total of 268 enhancement foci were found with CTHA in 50 patients, including 59 non-pathologic enhancement foci in 21 cases with an occurrence rate of 22.0%. The shape of non-pathologic enhancement was small round, irregular and wedge.

DISCCUSSION

In recent years, with the development of spiral CT technique, CTAP and CTHA have become widely used in diagnosis and differential diagnosis of small hepatocellular carcinoma. Compared with non-spiral CT arteriography, spiral CT arteriography has achieved great improvement not only in scanning technique, but also in quality of CT images[1]. We scanned the entire liver consecutively, and obtained excellent images of CTAP and CTHA by the technique of a single breath-hold scanning, with a comparatively small quantity of contrast material (CTAP 30ml-40ml, CTHA 20 ml - 30 ml). Compared with conventionally enhanced CT (contrast material administrated by venous injection), CTAP and CTHA have a high detectability of 80% - 96%[2]. In our 50 patients examined with CTAP and CTHA, the detectability rate of CTAP, CTHA and simultaneous use of both procedures were 92.2%, 90.1% and 94.8% respectively.

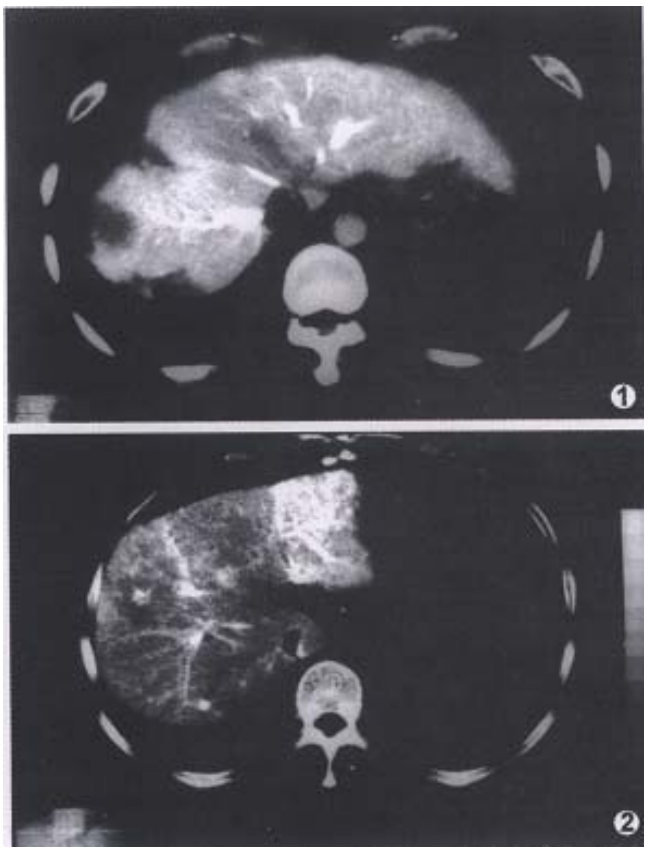

Figure 1 CTAP image obtained in a 42-year-old man with recurrence of HCC shows two tumors in right lobe. The peripheral wedge-shaped perfusion defect is non-pathologic perfusion defect, confirmed by biopsy.

Figure 2 CTHA image obtained in a 45-year-old man shows a small round peripheral enhancement in right lobe which is non-pathologic enhancement. No tumor is found in the operative pathology.

With the widespread use of CTAP and CTHA, there have been more reports on non-pathologic perfusion defects found with CTAP and non-pathologic enhancement with CTHA. The manifestation of pseudolesion in CTAP was noted relatively earlier. Due to aberration of blood supply for some parts of the liver, such as arterio-portal shunt[3], cirrhotic nodule, focal nodular hyperplasia, and focal fatty infiltration, the normal

tissue could show up as perfusion defects in CTAP, especially in the medial segment of the left lobe[4]. lrie *et al* reported that perfusion defects on CTAP are sometimes nonspecific, and suggested that peripheral flat or wedgeshaped perfusion defects indicate benignity. Their study showed that, except for cysts, perfusion defects larger than 1.5cm may indicate malignancy, and combination with CTHA might be helpful in differentiating malignant from benign perfusion defects with CTAP[5]. Peterson *et al*. reported that peripheral wedge-shaped perfusion defects on CTAP were nearly uniformly benign, and may be used as a criterion of benignity. They hypothesized that the pathogenesis of these peripheral wedge-shaped perfusion defects may represent variations in the normal portal perfusion of the microvasculature of the peripheral sub-capsular liver parenchyma[6]. In our series of 50 cases, the occurrence rate of non-pathologic perfusion defects on CTAP was 15.1%, and peripheral wedge-shaped perfusion defects were the most common (Figure 1).

Since the advent of helical CT technique, CTHA became more widely used in the differential diagnosis for hepatocellular carcinoma[7]. Similar to findings of CTAP, pseudo-lesion enhancement of normal liver tissue also occurred in CTHA. Kanematsu *et al*. supposed that local non-pathologic enhancement detected with CTHA might result from the cystic venous drainage or peripheral arterio-portal shunts. They analyzed the frequency, size, location, and shape of local non-pathologic enhancement on CTHA. In a series of 31 patients examined with CTHA, 36 non-pathologic enhancements were found in 14 cases, the occurrence rate being 36.4%. The shapes of non-pathologic enhancement on CTHA appeared round, veriform, irregular, punctate, and wedge-like[8]. Although the shape of the enhancement was nonspecific, Irie *et al*. suggested that rim enhancement indicated malignancy in both cirrhotic and non-cirrhotic liver[5]. In our study, the occurrence rate of non-pathologic enhancement on CTHA was 22.0%, and small rounded peripheral enhancement was the most common (Figure 2). In practice, we discovered that the frequency of non-pathologic enhancement was to some extent, related to the depth of catheterization at which CTHA was performed. There was a relatively higher frequency of non-pathologic enhancement in CTHA formed at catheterization of hepatic proper artery, compared with CTHA formed at catheterization of celiac artery trunk. The reason might be the relatively larger volume of contrast material and higher speed of injection in CTHA performed at catheterization of hepatic proper artery. Thus, we did not recommend CTAP or CTHA alone for interpretation of hepatocellular carcinoma. We suggest that the simultaneous use of both procedures help decrease the false positive rate of CTAP and CTHA, and increase the veracity of diagnosis for hepatocellular carcinoma.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):516-518

Diet and gastric cancer: a case-control study in Fujian Province, China *

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Subject headings stomach neoplasms/etiology; living habits; food habits; risk facto

Abstract

AIM To explore the relationship between consumption of fish sauce, other dietary factors, living habits and the rish kf gastric cancer.

METHODS From May 1994 **to July** 1995**, a population-based** 1:2 **case-control study was in Carried out in high-risk areas of gastric cancer, Changle and Fuqing cities, Fujian Province. Totally** 272 **cases and** 544 **age, gender-matched controls were included. Risk state analyses were made by ASRS package.**

RESULTS Risk state single-factor analysis indicated that gastric cancer risk rose with high intake of fish sauce (**OR=**2.57)**, salted vegetables** (**OR**=1.41)**, salted/fried fish and small shrimps** (**OR**=1.57)**, low consumption of fresh vegetables** (**OR**=1.95)**, fresh citrus fruits** (**OR** = 1.41)**, other fresh fruits** (**OR** = 1.31)**, green tea** (**OR**=1.72)**, exposure to moldy foods** (**OR**=2.32)**, irregular dinners** (**OR**=5.47) **and familial history of malignancy** (**OR**=3.27)**. No significant relationship was observed between smoking, drinking, salt intake, use of refrigerator and gastric cancer rish. The results of rish state conditional Logistic regression showed that fish sauce, salted dried fish and small shrimps, irregular dinners, familial history of malignancy were included in the best rish set. The summary ARS for the four factors was** 75.49%**.**

CONCLUSION High intake of fish sauce, salted foods, moldy foods, irregular dinners and familial history of malignancy were possible risk factors for gastric cancer, whereas fresh vegetables and fruits. and green tea might have protective effects for gastric cancer.

INTRODUCTION

Changle and Fuqing cities are located in the southeastern part of Fujian Province, China with a high incidence of gastric cancer. However, the causes of gastric cancer still remain unclear. Previous studies indicate that environmental factors may play an important role in the carcinogenesis of gastric cancer, among which, dietary rish factors for gastric cancer were most extensively investigated. Our hypothesis is that the high incidence of gastric cancer may be attributed, to some extent, to some unique dietary habits. Recently a statistically significant relationship between fish sauce consumption, a condiment commonly used by local residents, and the mortality rates from gastric cancer was observed by our ecological study[1]. The mutagenicity of fish sauce was also reported by experimental studies[2]. N-nitrosamines can also be detected in fish sauce[3]. In order to explore further the relationship between consumption of fish sauce and gastric cancer, a population-based 1:2 matched case-control study was carried out from May 1994 to July 1995.

MATERIALS AND METHODS

Selection of cases and controls

This study was conducted in Changle and Fuqing cities with populations around 600 thousands and 1 million, respectively. All cases histologically confirmed or diagnosed by operation from January 1993 to July 1995 were collected from cancer registry and a quick-reporting system from hospitals. Each case was matched by two randomly selected controls who resided in the same village as index case, with same gender, nationality and age (±3 years). Those who have ever been diagnosed having gastric diseases within the past 3 years were not eligible as controls. Study subjects must have

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Received 1998-06-06

resided in the two cities for more than 20 years, and can answer questions clearly.

Investigation

Face to face interviews were made by specially trained interviewers with a structured questionnaire. The items of questionnaire included demographic and socio-economic factors, occupational and medical histories, family occurrence of cancer, use of alcohol and tobacco, and dietary habits. As for dietary habits, we emphasized on the exposure 20 years before. Diet was assessed with a food frequency questionnaire. The subjects were interviewed about the usual frequency of consumption of certain foodstuffs, supplemented with questions about the actual amount consumed per time unit. Then total amount per year was estimated accordingly. The subjects within a matched pair were interviewed by one interviewer.

Statistical analysis

Data werre handled by Epi-info. The statistical analyses, including univariate analysis and conditional logistic analysis were done using ASRS software[4]. In univariate analysis, the best cut-points for exposure levels were searched by automatic or forced adjustment, combination, on the criteria of CPDS ans AIC. Those factors, which showed association with the risk of gastric cancer in univariate analysis, were further investigated by conditional logistic analysis of risk states to establish a main-effect model.

RESULTS

Totally 272 pairs were investigated, among which 157 were from Changle City, 115 from Fuqing City; 233 were male and 39 female. The age range for cases was 30-78 years, averageing 58.67 years. No obvious difference in marital status, occupation and education level was observed between cases and controls.

The results of univariate analysis are shown in Table 1. As for dietary habits, consumption of fish sauce, salted vegetables, salted fermented sea products and moldy foods may increase the rish of gastric cancer. However, fresh vegetables, fruits and green tea may have protective effects against gastric cancer. Additionally, irregular dinner and family occurrence of cancer are also risk factors for gastric cancer. No association between the use of alcohol and tobacco, the amount of salt intake, the use of refrigerator and the risk of gastric cancer was observed. To select a possible best subset of risk factors for gastric cancer, conditional logistic analyses of risk states were also made. The results showed that the best subset of risk factors included fish sauce, irregular dinner, salted fermented sea products and family occurrence of cancer (Table 2). The summary attributable risk for these four risk factors is 75.49%, indicating that these four factors may play an important role in the carcinogenesis of gastric cancer.

Table 1 Results of univariate analysis on the relationship between fish sauce, other factors and the risk of gastric cancer

Factors	Exposure	Case	Control	OR	95%CI
Index of smoking[a]	>10	118	241	1	
	≤10	154	303	1.04	0.95-1.13
Hard distilled spirit	≤20kg/y	265	534	1	
	>20kg/y	7	10	1.41	0.63-3.14
Soft distilled spirit	>25kg/y	13	29	1	
	≤25kg/y	259	515	1.12	0.86-1.47
Wine	≤30kg/y	245	494	1	
	>30kg/y	27	50	1.09	0.89-1.33
Beer	>50 bottle/y	42	106	1	
	≤50 bottle/y	230	438	1.33	0.93-1.88
Green tea	>0.75kg/y	47	144	1	
	≤0.75kg/y	225	400	1.72	1.26-2.36[e]
Fish sauce	<0.4kg/m	198	475	1	
	≥0.4kg/m	74	69	2.57	1.89-3.50[e]
Salt[b]	≤0.25kg/m	159	347	1	
	>0.25kg/m	113	197	1.25	0.96-1.63
Moldy foods	No	188	456	1	
	Yes	84	88	2.32	1.73-3.09[e]
Irregular dinners	<3 times/w	114	434	1	
	≥3 times/w	158	110	5.47	4.22-7.09[e]
Use of refrigerator	Yes	35	78	1	
	No	237	466	1.13	0.85-1.52
Salted vegetables	<2kg/y	157	358	1	
	≥2kg/y	115	186	1.41	1.09-1.83[d]
Salted fermented sae foods	<1.5kg/y	144	347	1	
	≥1.5kg/y	128	197	1.57	1.21-2.02[e]
Citrus fruits-c	>2.5kg/y	55	143	1	
	≤2.5kg/y	217	401	1.41	1.03-1.92[d]
Other fruits	>2.5kg/y	166	366	1	
	≤2.5kg/y	106	178	1.31	1.01-1.71[d]
Fresh meat, fish, egg, poultry	>25kg/y	105	238	1	
	≤25kg/y	167	306	1.24	0.95-1.61
Fresh vegetables	>25kg/y	212	475	1	
	≤25kg/y	60	69	1.95	1.41-2.70[e]
Family occurrence of cancer	No	165	454	1	
	Yes	107	90	3.27	2.48-4.31[e]

[a]smoking index: (amount of smoking/day x years of smoking) / age of starting smoking; [b]including the salt in fish sauce and soybean sauce; [c]including orange, grapefruit, banana; [d]$P < 0.05$; [e]$P<0.01$.

Table 2 The results of conditional logistic analysis of risk states

Factors	Regression coefficient	Standardized regression coefficient	Adjusted attributable risk[a]
Fish sauce	1.08	3.49	17.81%[c]
Irregular dinners	1.85	8.26	48.93%[c]
Salted sea foods	0.54	2.11	19.69%[b]
Familial history of malignancy	1.19	5.45	27.41%[c]

[a]Comprehensive attributable risk (CAR) = 75.49%; [b]$P < 0.05$; [c]$P<0.01$.

DISCUSSION

Fish sauce is one kind of condiments consumed daily by local residents. It is produced by long-term fermentation from several kinds of sea frsh. Due to the proteins with amino in the fishes, salted fermented fish products may contain a large amount of important precursors of N-nitroso compounds-amines[5]. These precursors may react with nitrite in gastric juice to form N-nitroso compounds internally. Deng et al reported that abstracts of fish sauce from Changle have carcinogenicity and mutagenicity after nitration[2]. The amount of N-nitro compounds increased greatly after nitration, and genotoxins can be detected[3]. Our ecological study indicated that there was a statistically significant relationship between frsh sauce consumption and mortality rates from gastric cancer among 14 counties in Fujian Province[1]. The results of this study further supported the point that fish sauce consumption may be an important cause for the high incidence of gastric cancer.

Several case-control studies have indicated that long-term use of refrigerators may decrease the risk of gastric cancer[6-8]. However, in our study, refrigerators were not commonly used by local residents. Even among those users, the history of refrigerator use is very shout. Therefore, it is impossible to evaluate the role of refrigerator use in the etiology of gastric cancer. However, the deficiency of refrigerator indicated that the consumption of salted foods was very common. Especially in the study area which is located on seaboard, the consumption of salted or fermented sea foodstuffs is very common. In our study, consumption of salted foodstuffs, especially of salted fermented sea products was found to increase the risk of gastric cancer. This is in accordance with the results of Buiatti *et al*[7]. Additionally, our study indicated that irregular dinner may be one of etiological factors for gastric cancer. This finding supported the results of our previous ecoloical study[1]. Irregular dinner may cause injuries of gastric mucosa and promote the effects of carcinogens. A prospective study showed that those with familial history of malignancy, especially gastric cancer, havea higher risk of gastric cancer[9]. This is in agreement with our study results. In our study, fresh vegetables showed a protective effect against gastric cancer. This is also true for citrus fruits and other fruits. Their protective effects may be attributed to the vitamin C, which may interrupt the internal formation of N-nitroso compounds. Some studies reported that only consumption of raw vegetables had a protective effect[6,7]. However, in our study area, residents do not have such a habit. There were conflicting views on the relationship between the use of alcohol and tobacco and the risk of gastric cancer[6,7,9,10]. In our study, no association was observed between the use of alcohol, tobacco and the gastric cancer risk. No interaction between these two factors was found either.

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Experimental study on mechanism and protection of stress ulcer produced by explosive noise

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Subject headings stomach ulcer/etiology; stomach ulcer/prevention and control; gastric mucosa/pathology; noise/advers effects; stress ulcer

Abstract

AIM To establish an experimental model of stress ulcer produced by explosive noise, and to probe into its mechanism and protection.

METHODS The country standard Wistar white rats were randomly divided into control group (*n* =8), which were neither stimulated nor protected, and stimulating group (divided into subgroups A, B and C, including 8 rats each which were decapitated to draw blood for test immediately, 12 hours and 24 hours after stimulation) and prevention group (divided into subgroups A, B and C, having 8 rats each, subgroup A was given cimetidine, B anisodamine and C both drugs). Firing noises of submachine guns were used as inflicting factor. The rats were fasted for 24 hours and stimulated by firing noise for 12 hours. The change of ulcer index, gastric mucosal and related serum hormones were observed.

RESULTS Stress ulcer was significant in the stimulating group, and its ulcer index (8.6±0.6) was remarkably higher than that in both the control group and prevention group (0.3±0.1, *P* <0.01). Its serum gastrin (Gas ng/L, 294±163 *vs* 63±40, *P*<0.01) and endothelin (ET ng/L, 181±57 *vs* 135±42, *P*<0.1) were apparently higher than those in the control group, and its serum nitric oxide (NO) level was conspicuously lower than that in the control group (ng/L, 0.2± 0.1 *vs* 0.8 ± 0.5 *P* < 0.5), while the serum gastrin level (ng/L, 556±225) in prevention group was distinctly higher than that in both the control (*P*<0.01) and stimulating group (*P*< 0.05). There were no significant differences in the changes of ET and NO between the control and the stimulating groups.

CONCLUSION Stress ulcer model of rats can be successfully established by the stimulation of explosive noise. Gas, ET and NO are related to the formation of stress ulcer, and play an important role in its mechanism. Hepatic function affected by noise is observed in this experiment.

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Received 1998-05-18

INTRODUCTION

Explosive noise may produce an enormous adverse effect on human body and mind. It has been known that peptic ulcer occurred much more frequently in war time than in peace time[1]. However, there have been no reports about the relationship between explosive sound and stress ulcer in literature. The aim of this study is to establish an animal model of stress ulcer produced by explosive noise and probe into its mechanism, prevention and treatment.

MATERIALS AND METHODS

Materials

The healthy country standard Wistar rats were provided by the Animal Center of the Fourth Military Medical University. Submachine guns, a precision pulse counter, and a frequency spectrum analyser were provided by the Chinese PLA 371 Hospital. A PJ-2003/50 G-type γ radioimmunity counter (made in Xi'an), 754 spectrophotometer (made in Hefei), a high-speed and low-temperature centrifuge (made in Tumen), a light microscope and a JEM-2000EX-type transmission electron microscope (made in Japan) were used in this experiment.

Methods

Preparation of ulcer model. Having been fasted for 24 hours, the experimental rats were confined in an isolated room to be tested. Firing noise of submachine guns acted as an inducing factor which had been recorded on a tape and was played to the rats through a loudspeaker at a distance of 20cm-30cm. Examined with the precision pulse counter and the frequency spectrum analyser, the intensity of the firing noise was measured as 110dB(A) and its frequency as 0.25kHz-4.00kHz. After the rats were stimulated successively for 12 hours by gun

shot sound, their stomachs were opened and the formation of their stress ulcer was observed.

The rats were randomly divided into three groups: ① Control group, consisting of 8 rats, which were neither stimulated nor protected; ② stimulating group, subdivided into groups A, B and C, including 8 rats each. These subgroups were decapitated to draw blood for test immediately, 12 hours, and 24 hours after stimulation respectively; and ③ prevention group, also subdivided into groups A, B and C, having 8 rats each. Group A was injected 40mg ip with cimetidine 30min before the stimulation; group B given 2 mg ip of anisodamine 30min before and 6 hours after the stimulating; group C administered with above mentioned combined drugs. All the rats used in this study were anesthetized in their abdominal cavities with 1.2mg of 5g/L amobabital sodium after treatment and their abdomens were opened for observation on the changes of their gastric mucosa. After decapitation, two blood samples were taken: one for centrifugation of serum, the other added with EDTA and retardant peptidase for separation of plasma and kept at -40℃ in a refrigerator ready for the detection of hormone and hepatic and renal functions.

Measurements of mucosal damage. According to Guth's method[2], the length of gastric mucosal damage area <1 mm was scored 1 point; 1mm-2 mm 2 points; 2mm-3mm 3 points; 3 mm - 4 mm 4 points, >4 mm scored segmentally. The sum total of the scores of the whole stomach constituted the ulcer index. Tissue and cell structures of all groups were observed and photoed under light microscope and electroscope respectively.

Detection of plasma Gas, ET, NO and hepatic-renal functions. Gas was detected before and after stimulation using the kit produced by the Northern Immunity Agents Research Institute in accordance with the instructions. ET was detected using the kit by the East Asian Immunity Technique Research Institute according to the operational manual. NO was tested by means of the kit provided by the Military Medical Academy according to the manual. Hepatic and renal functions were tested by the laboratory from the serum samples we sent.

Statistics. Both t test and analysis of variance were used for this study.

RESULTS

Observation of gross specimens

In control group, after the gastric wall was opened, the mucosa was seen intact, tidy and smooth without any ulcer or erosion except for a couple of bleeding spots under the mucosa of 2 rats.

In stimulating group, on the abdominal cavity, different degrees of congestion and edema were found in seruous layers and on the gastric walls, congestion, edema, erosion and ulcer formation came into sight (Figure 1), especially in group B (ulcer index, 8.4 ± 0.6), the ulcer index being remarkably different ($P < 0.01$) between group B and the prevention group (ulcer index, 0.3 ± 0.1) and control group (ulcer index, 0).

In prevention group, although there were still some congestion, edema, erosion and ulcer formation, these were conspicuously lower than in the stimulating group ($P < 0.01$). The preventive effects proved significantly better in group C' (0.3 ± 0.1) than those in groups A' and B'($P<0.05$).

Examination under light microscope

In Control group, mucosal layers were smooth and tidy, glands were arranged in order, and no tendency of inflammatory cell infiltration and submucosal hemorrage appeared. In stimulating group, in sub gropus A, B and C, there were interruptions of mucosa, enlarged glandular gaps and damaged parts of gland. Meanwhile, a large amount of RBCs were accumulated among the glands. Within submucous eosinophil, infiltration and capillary thrombosis were detected (Figure 2). In prevention group, all of the 3 subgroups were found intact in mucos, well-structured in glands slightly broadened in gaps with some RBCs sparsely existing beneath mucosa.

Examination under electron microscope

The control group showed intact cell structure, regular secretory granules, and no widened gaps among nuclei. In addition to these, mitochondrion and endoplasmic reticulum were also conspicuous. The structure of microvillus was perfect. On the contrary, the stimulation group exhihited irregular cell structure, with nuclei withered, gaps among nuclei broadened, endoplasmic reticulum expanded, mitochondrion hypertrophic, inflammatory cell infiltration in interstitial, and secretory granuals increased (Figure 3). The prevention group in appeared differently. Although there were slight withered nuclei, broadened nuclear gaps, and more or less expanded endoplasmic reticulum, all these were noticeably insignificant as compared with those in the stimulating group (Figure 4).

Blood biochemical examination

Gas was apparently higher in the stimulating and prevention groups than in control group ($P<0.05$, Table 1), being most pronounced in stimulating group B.

ET was obviously higher in the stimulating group than in control group, while there were no significant changes between the prevention and control groups ($P<0.05$), nor between stimulating

and prevention groups (P<0.05).

NO was lower in the stimulating group than in control group (P<0.05), especially in group C (Table 1). No substantial difference was seen between the prevention and control groups (P<0.05). ALT and BUN showed no remarkable changes among all groups (P<0.05), while AST was found higher in the stimulating and prevention groups than in controls (P<0.05, Table 2).

Table 1 Serum Gas, ET, NO levels in stress rats (n=8, $\bar{x}$±s, ng/L)

	Controls	Stimulating group			Prevention group		
		A	B	C	A'	B'	C'
Gas	63±40	315±193[a]	294±163[a]	70±8	327±93	429±266[a]	556±225[b]
ET	135±42	177±87	181±57[a]	169±26	157±37	158±55	139±43
NO	0.8±0.5	0.5±0.2	0.2±0.1[a]	0[b]	0.4±0.3	0.7±0.5	0.7±0.5

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

Table 2 Serum ALT, AST, BUN level in stress rats (n = 8,$\bar{x}$±s, ng/L)

	Controls	Stimulating group			Prevention group		
		A	B	C	A'	B'	C'
ALT mmol/s	867±67	867±183	997±133	867±117	997±116	804±184	834±100
AST mmol/s	2084±183	2834±583[a]	2501±450	2450±467	2634±233[a]	2384±367	2317±283
BUN mmol/L	7.8±0.8	8.4±2.1	7.7±1.5	12.2±1.9	11.5±2.7	8.3±2.1	9.2±1.4

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

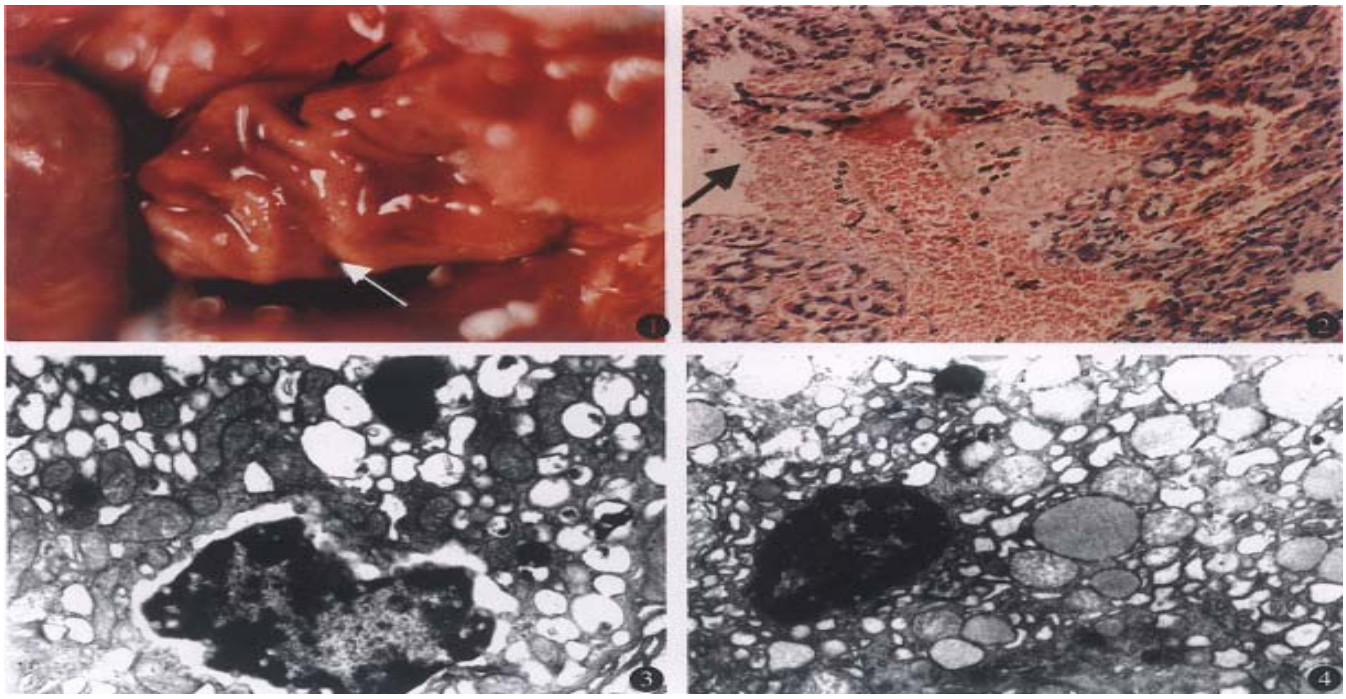

Figure 1 Gross specimens in stimulating group congestion, edema, erosion and ulcer formation.

Figure 2 Stimulating group: interruptions of mucosa, enlarged glandular gaps, damaged parts and a large amount of RBCs accumulated among glands. HE×10

Figure 3 Stimulating group exhibited irregular cell structure, with nuclei withered, gaps among nuclei broadened, endoplasmic reticulum expanded, mitochondrion hypertrophic, granuals increased. EM×7500

Figure 4 Prevention group: slight withered nuclei, a little broadened nuclear gasps and more or less expanded endoplasmic reticulum. EM×6000

DISCUSSION

There are five types of stress ulcers[3], i.e., single bound stress, cold bound stress, socking stress, shock stress and spinal cord injury stress. It has been reported that noise may damage the human body and mind by causing disturbance in the stomach and intestines[4] and that ulcer occurred conspicuously more frequently in war time than in peace time[1]. Based on these findings and considering that rats are characterized by well-developed adrenalin function, sensitive response to stress and susceptiveness to stress ulcer[5], we designed and prepared the stress ulcer model of rats in order to explore its mechanism and prevention and treatment. Our results showed that a typical ulcer model of rats can be successfully established by stimulation with a strong explosive sound continuously for 12 hours. Using Guth's methods[2], the ulcer index was found to be significantly different between stimulating group and both the control group and the prevention group ($P<0.01$). Such changes as interruptions of mucosa and damage of glands were detected under light microscope (Figure 1). This, therefore, has opened a new path to establish an animal model of stress ulcer.

In an attempt to probe into the underlying causes, we laid special stress on analysing the role of ET, NO and Gas in this disease. To our knowledge, ET serves as a strongest vasoconstrictive substance, while NO functions as a vasodilative one. They are contradictory regulating factors in the blood[6]. When balance between the two is lost, the blood flow in gastric mucosa may change and an acute mucosal damage occurs. ET has potent ulcerogenic and vasoconstrictive actions in the stomach where it induces gastric mucosal damage and increases gastric vascular tone[7]. In our study, after sound stimulation, serum ET level became conspicuously higher in stimulation group than in control group ($P<0.05$), indicating ET played an important part in stress ulcer. This was probably because the gun-shot sound overexcited the sympathetic nerves to cause submucosal capillaries to constrict, thus stimulating endodermical cells to produce excessive ET, which, in turn aggravated vasoconstriction and decreased blood flow under mucosa, and, finally, resulted in the erosion and formation of ulcer. NO is produced when larginine guanidine combined with oxygen is converted intolcitriline under the catalysis of nitric oxide synthetase (NOS), which is the key factor for the production of NO. Kanno *et al*[8] reported that an induced NO (iNOS) can be brought about by a variety of cell factors and lipopolysaccharide (LPS) in vascular endothelia and smooth muscles and then produce more and more NO. However, in case of stress, because of vasoconstriction of gastric mucosa and decrease of blood flow, the production INOS in vascular endothelia can be inhibited, leading to lowered NO level. In addition, erosion and bleeding in mucosa after stress can also cause the synthesized NO to be inactivated by combining with hemoglobin[9], thereby lowering NO level. The decrease of NO level, together with the weakening of mucosal protection and vasodilation, further aggravates erosion of mucosa and formation of ulcer. Our results showed that NO level was noticeably lower in the stimulating group, especially in group C than in control group, which conforms to their findings. After stimulation by explosive noise, with sympathetic nerves overxcited, vagus nerves inhibited[10], cortisol secretion is, of course, increased, resulting in dysfunction of peptidergic nerves in non-adrenergic and non-cholinergic nerves within sympathetic and parasympathetic nerve systems[11], thereby, it causes G-cells to secrete more and more Gas. This may stimulate wall cells to produce excessive gastric acid, pH value in stomach lowered, and H^+ back oozing, leading to inflamation, congestion, edema, erosion in gastric mucosa and even the formation of ulcer. Our results showed that Gas was apparently higher in the stimulating group than in control group ($P<0.05$). It was discovered under dynamic observation that Gas secretion increased gradually after treatment, reached its peak at the 12th hour, and then returned to normal after 24 hours. All these indicated that Gas not only took part in but played an important role in the process of formation of stress ulcer.

Explosive noise also exerts some harmful effects upon functions of the liver and kidneys. Liu *et al*[10] noted that following stimulation by noise, the tension of sympathetic nerves became stronger, and tissue metabolism exuberated, accompanied by vasospasma and tissue ischemia, with the result that the renewal of liver cells hastened and ALT of blood increased. Our results were in agreement with theirs, but insignificant in statistics. This may be due to limited number of samples. AST was remarkably higher in the stimulating group, especially higher group A than in control group ($P<0.05$). However groups B and C showed a gradual decline. The reason why AST became higher may be that during the stress the sympthetic nerves of rats were so excited that their muscles all over the body kept constricting, and their hearts beat faster for a short time, which caused damages in their skeleton muscle and cardiac muscle cells, and led to the increase of AST release. After removal of stimulation, these actions weakened, cell functions recovered and AST in blood decreased gradually.

The strong noise for a short time, therefore, did little damage to the function of the kidneys.

Our study also proved that among the three prevention groups, group C' for which drugs were given in combination brought best results. Next to this were groups B' and C' treated with anisodamine and cimetidine respectively. Measured by analysis of varience, their ulcer index was significantly different from group A' (P<0.05), corresponding to the previous documents. Following medication, plasma Gas leve rose more remarkably in the prevention group than in the stimulating and control groups. This might be related to the fact that after using H2 receptor-antagonist and vasodilator agents, the secretion of gastric acid by wall cells was inhibited, and by feedback the secretion of Gas by G-cells at gastric antrium increased. Serum NO and ET did not change conspicuously before and after medication (P>0.05), presumably because H_2 receptor-blocking agents and vasodilator agents reduced the secretion of gastric acid, and abated the stimulation to constriction of submucous blood vessel. On the other hand, it brought about dilatic factors in capillaries, thus the releasing of ET by endodermical cells was weakened. As a result, the producing effect of iNOS was also suppressed so that the formation of NO remained unaffected. Another reason why ET and NO level stayed unchanged was supposedly that the alliviated mucosal erosion and ulcer reduced the mucosal bleeding and combination of NO with hemoglobin.

From these results, we came to the conclusion that although explosive noise can stimulate the production of stress ulcer, it can be effectively prevented and cured by such medicine as H_2 receptor antagonist and vasodilator agents. Therefore, we believe that use of preventive medicines before war will be of significance for diminution and protection of stress ulcer in wartime.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):524-526

Effect of devazepide reversed antagonism of CCK-8 against morphine on electrical and mechanical activities of rat duodenum *in vitro* *

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Subject headings duodenum; devazepide; morphine; electrophysiology; cholecystokinin octapeptide

Abstract

AIM To study the antagonism of cholecystokinin octapeptide (CCK-8) against the effect of morphine and its mechanism.

METHODS The method and mechanical activities of rat duodenum *in vitro* were recorded simultaneously.

RESULTS Acetylcholine (ACh) could increase the amplitude and the number of the spike potential (SPA and SPN) of rat duodenum *in vitro*, followed by the increase of the duodenal contraction amplitudes (CA), showing a positive correlation. Morphine, on the contrary, inhibited the potentiation of ACh, showing a negative correlation. CCK-8 could antagonize the effects of morphine, i.e. th SPA and SPN were increased again, followed by the increase of CA. CCK-A receptor antagonist Devazepide could reverse the antagonism of CCK-8 to the effect of morphine.

CONCLUSION CCK-8 could antagonize the effect of morphine which inhibited the potentiation of ACh on the duodenal activities *in vitro*. The antagonistic effect of CCK-8 on morphine was mainly mediated by CCK-A receptor.

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*Supported by the Scientific Technological Foundation of Provincial Education Committee of Heilongjiang in China, No.95-8.

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Received 1998-07-06

INTRODUCTION

Cholecystokinin octapeptide (CCK-8) is a typical brain-gut peptide. Many data show that CCK-8 has been the strongest endogenous anti-opioid substance up to now. Faris pointed out that CCK-8 could block morphine analgesia in the rat tail flick test[1]. Han[2] and Xu[3] reported respectively that CCK-8 antagonized the analgesic effects of morphine and electroacupuncture (EA), and played an important role in the induction of morphine tolerance and EA tolerance using the behavioral changes and electrophysiological methods. Zetler indicated that morphine and opioid peptides antagonized the hyperfunction of contraction of guinea-pig ileum *in vitro* induced by CCK-8 like peptide[4]. Valeri proved that endogenous opioid peptides could antagonize the effects of CCK-8, in the similar experiment[5]. But few report about the anti-opioid effect of CCK-8 on the duodenum *in vitro* was found.

In this experiment, the method of simultaneously recording the electrical and mechanical activities of rat duodenum *in vitro* was adopted so as to inquire into the antagonism of CCK-8 to the effect of morphine and its mechanism.

MATERIALS AND METHODS

Experimental animals

Twenty-five Wistar rats (Grade II, 195 g - 295 g), Animal Department of Tumour Institute of Heilongjiang Province, were used.

Experimental animals

Tyrode's solution (made by ourselves); Acetylcholine (ACh, 300nmol/L, Shanghai Third Reagent Factory, China); morphine hydrochloridum (330 nmol/L, Shenyang First Pharmaceutical Factory, China); CCK-8 (0.7nmol/L, Squibb, USA) ; Devazepide (10nmol/L, Merck Sharp and Dohme Researched Laboratories, USA).

Experimental method

A rat was given peritoneal anaesthesia with 20%

urethan (5ml/kg), the peritoneum was opened and one or two segments of 2cm duodenum were cut off under pylorus. The duodenal segments were put into a bathtube containing 50ml Tyrode's solution which was 38¡æ and saturated with oxygen. Then the bathtube was maintained at 38℃ in CS-501 superthermostat. One end of duodenal segment was fixed with resting load of 5g and the other end was connected with a LZ-1 tension transducer according to longitudinal axis of jejunum. Thus contractions of duodenal smooth muscle were recorded. The electrical activities of duodenum were led out by silver adsorptive electrode. Through bioelectrical amplifier, the electrical activities, mechanical contraction and time scale were simultaneously recorded by ST-41 multipurpose polygraph. The parameters were modulated as follows: time constant 0.3 second, high frequency wave filter 30Hz, electrical gain 3, mechanical gain 4, recording paper velocity 5 mm/s[6].

Firstly, normal electrical and mechanical activities of every segment of duodenum was simultaneously recorded. Then 200μl of ACh was injected quickly into the bathtube by a microinjector. Sixty seconds after the injection of ACh, 50μl of morphine hydrochloridum was administered. At 120 seconds and 240 seconds, 40μl CCK-8 and 20μl Devazepide were added respectively.

Statistical analysis

Each value was expressed as $\bar{x}$±s. All data were analyzed with paired *t* test.

RESULTS

CCK-8 antagonized the inhibition of morphine to the effect of ACh

Before the injection of ACh, the amplitudes and numbers of the spike potential (SPA and SPN) of 44 duodenal segments and their corresponding contraction amplitudes (CA) respectively averaged 0.70 ± 0.04 mV, 2.41 ± 0.13 and 16.58 mm ± 0.65 mm. At 60 seconds after the injection of ACh, the SPA, SPN and corresponding CA increased to 0.97mV ± 0.05 mV, 2.46 ± 0.11 and 24.50 mm ± 0.99mm, respectively. At this time, morphine was administered. The SPA, SPN and CA decreased to 0.63mV ± 0.04 mV, 2.38 ± 0.08 and 14.73 mm ± 0.69mm respectively at 60 seconds after the injection of morphine. At 120 seconds after adding CCK-8, the SPA, SPN and CA increased to 0.84 mV ± 0.04 mV, 3.29 ± 0.09 and 22.77 mm ± 0.68 mm, respectively. Moreover, all of them showed significant differences (P<0.01) when the latter was compared with the corresponding item of the former (Figure 1).

Devazepide reversed the antagonism of CCK-8 to the effect of morphine

Figure 2 showed the electrical and mechanical change curves of one segment of duodenum simultaneously recorded by multipurpose polygraph. It indicated that the SPA and SPN of the duodenal segment increased, followed by the increase of the CA after the injection of ACh, showing the enhancement of duodenal activities. Whereas the SPA, SPN and corresponding CA were reduced when morphine was administrated, showing that morphine inhibited the excitatory effects of ACh. At this moment, the injection of CCK-8 increased the SPA, SPN as well as CA, suggesting an antagonism of morphine effect by CCK-8. On the basis of the above, after CCK-A receptor antagonist Devazepide was administrated, the SPA and SPN were decreased again, accompanied by the reduction of CA. It showed that Devazepide reversed the anti-morphine effect of CCK-8. Moreover, every contraction wave occured after the beginning of spike potential over the slow potential and the ratio between slow potential and contraction wave was 1:1.

The statistical analytical results of 22 duodenal segments are shown in Table 1.

Table 1 Anti-morphine effect of CCK-8 reversed by Devazepide

Items	Control n = 22 (0s)	Ach 300nmol/L (60s)	Morphine 330nmol/L (120s)	CCK-8 0.7nmol/L (240s)	Devazepide 10nmol/L (325s)
SPA(mV)	0.70±0.07	0.94±0.09[b]	0.59±0.06[d]	0.81±0.07[f]	0.54±0.05[h]
SPN	2.71±0.23	3.88±0.15[b]	2.71±0.09[d]	3.52±0.13[f]	2.66±0.18[h]
CA(mm)	16.40±1.00	24.44±1.63[b]	13.54±1.04[d]	22.73±1.00[f]	13.67±0.66[h]

[b]P<0.01 (ACh *vs* control); [d]P<0.01 (morphine *vs* ACh); [f]P<0.01 (CCK-8 *vs* morphine); [h]P<0.01 (Devazepide *vs* CCK-8).

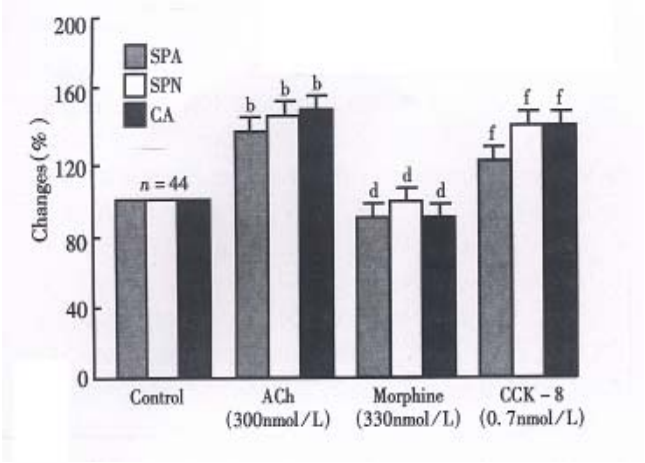

Figure 1 Elimination of inhibited effect of morphine on ACh by CCK-8.

[b]$P<0.01$ (ACh *vs* control); [d]$P<0.01$ (morphine *vs* ACh); [f]$P<0.01$ (CCK-8 *vs* morphine).

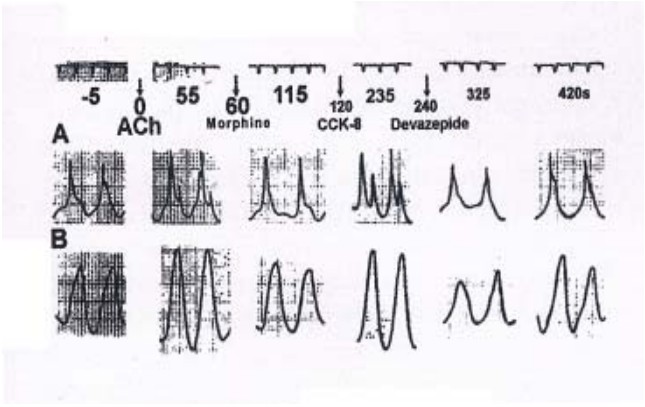

Figure 2 Anti-morphine effect of CCK-8 reversed by Devazepide.

A: electrical activity; B: mechanical contraction; ↓: drug injection; Time scale: 1s.

DISCUSSION

CCK-8 was the first brain-gut peptide found in human. CCK-8 existed in brain and peripheral tissues of animals and human[7]. Previous works indicated that an antagonistic interaction might occur between CCK and opioid peptides. Our experimental results demonstrated that CCK-8 per second did not show any effect, but could selectively antagonize the effects of morphine which inhibited the potentiation of ACh to rat duodenum *in vitro* with the electrical and mechanical activities. The conclusion was similar to those previous reports[4,5].

Recent receptor binding studies have confirmed the existence of 2 distinct CCK receptor subtypes, i .e. CCK-A and CCK-B receptor were present in both brain and peripheral tissues[8]. Devazepide was considerably more potent in inhibiting CCK binding to peripheral-type receptor (CCK-A) than to brain-type receptor (CCK-B)[9]. This results showed that Devazepide could reverse the antagonism of CCK-8 to the effect of morphine, therefore it was inferred that CCK-A receptor participates in the anti-morphine effect of CCK-8.

To sum up, our present work firstly demonstrated that CCK-8 could antagonize the elimination of morphine on the potentiations of ACh to duodenal activities, and these effects were mediated by CCK-A receptor. It is suggested that CCK-like peptides and opioid substances together with cholinergic system could regulate the gastrointestinal activities, and provided a new experimental basis for further research in the clinical treatment of the intestinal motility disturbances.

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Study on the causes of local recurrence of rectal cancer after curative resection:analysis of 213 cases

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Subject headings Rectal neoplasms/surgery; rectal neoplasms/pathology; neoplasm recurrent, local

Abstract

AIM To study the local recurrent rate and the causes of rectal cancer after surgery.

METHODS The clinicopathological data of 213 rectal cancer patients and the follow-up information were analyzed. The overall recurrent rate and the recurrent rates from different surgical appreaches were calculated. The main causes of recurrence were investigated.

RESULTS Among the 213 cases, 73 (34.27%) had local recurrence. The recurrent time ranged from 3 months to 62 months after the first operation. Most of the recurrence (65/73, 89.04%) occurred within 3 years after operation.

CONCLUSION Local recurrence had no significant correlation with surgical methods or pathological types, but closely related to Dukes' stages, location of primary tumors and the length of the distal rectum resected. Early resection and a wide tumor free resection margin are key factors to prevent local recurrence.

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Received 1998-06-06

INTRODUCTION

For rectal cancer, surgical resection remains the only possible cure. However, long-term survival after surgery is not satisfactory due to local recurrence or distant metastasis. Local recurrence is a major cause of cancer-related morbidity and mortality. To evaluate the rate and find out the causes of local recurrence after radical resection for rectal cancer, we carried out the following study.

PATIENTS AND METHODS

We studied 213 successive patients (108 males and 105 females) aged 21 to 78 years who underwent curative surgery for rectal cancer between January 1986 to January 1993, in the Institute of Oncology, Hubei Medical University. Primary tumor sites in this series were in the upper segment of their rectum (28), in the middle segemnt (52), and in the lower segment (133). The pathological types were papillary adenocarcinoma in 33 cases, tubular adenocarcinoma in 121 cases, mucinous adenocarcinoma in 30 cases, villous adenocarcinoma in 10 cases, signet-ring-cell carcinoma in 9 cases, and undifferentiated carcinoma in 10 cases. According to the Dukes' staging system, 50 cases were in stage A, 88 in stage B, and 75 in stage C. The initial operation procedures were Miles operation in 108 cases, Dixon operation in 86 cases and Bacon operation in 19 cases. The recurrence was confirmed by digital rectal examinatiion, ultrasonography, computer tomography (CT) scan, biopsy and pathology, if necessary.

Statistical analysis

The Chi-square analysis was employed on computer using SAS software to evaluate the difference among different categories, with P=0.05 as the level of significance.

RESULTS

Overall rate

Among the 213 cases, 73 (34.27%) had recurrence.

Time of recurrence

Recurrence within 3 to 24 months after operation happened in 37 cases, within 25 to 36 months in 28

cases, and over 37 months in 8 cases. Most of the recurrence (65/73, 89.04%) occurred within 3 years after operation.

Site of recurrence

Thirty-five cases recurred in the pelvic cavity, 21 in the anastomosis, 16 in the perineal region and 1 in the abdominal incision.

Pathological types and recurrence

The rates of recurrence were higher in mucinous adenocarcinoma and undifferentiated carcinoma than in villous adenocarcinomna, tubular adenocarcinoma, papillary adenocarcinoma and signet-ring-cell carcinoma, although the difference was of no statistical significance ($P > 0.05$, Table 1).

Table 1 Pathological types and local recurrence

Pathological types	Number	Local recurrence (%)
Tubular adenocarcinoma	121	40 (33.06)
Papillary adenocarcinom	33	11 (33.33)
Mucinous adenocarcinoma	30	12 (40.00)
Villous adenocarcinocna	10	3 (30.00)
Undifferentiated carcinoma	10	4 (40.00)
Signet-ring-cell carcinoma	9	3 (33.33)
Total	213	73 (34.27)

Operational approaches and local recurrence

The rates of local recurrence in Miles, Dixon, and Bacon operation were 37.01%, 31.40% and 31.58%, respectively. The difference was of no statistical significance ($P > 0.05$, Table 2).

Table 2 Operational methods and local recurrence

Operationa methods	Number	Local recurrence (%)
Miles	108	40 (37.01)
Dixon	86	27 (31.40)
Bacon	19	6 (31.58)
Total	213	73 (34.27)

The length of distal rectum resected in Dixon operation and anastomotic recurrence

Among the 86 patients who underwent Dixon operation, 27 had local recurrence, 21 of which were anastomotic recurrence. In the 26 cases with a distal resection margin of less than 3 cm, 11 (42.31%) had anastomotic recurrence. However, in the 60 cases with a distal resection margin of greater than 3 cm, only 10 (16.67%) had anastomotic recurrence. The difference was statistically significant ($P < 0.05$, Table 3).

Table 3 Distal resection margin in Dixon operation and anastomotic recurrence

Length of distal resection margin	Number	Anastomotic recurrences (%)
<3cm	26	11 (42.31)
⩾3cm	60	10 (16.67)[a]
Total	86	21 (24.42)

[a]$P<0.05$ *vs* <3cm margin group.

Dukes' stages and local recurrence

The rates of local recurrence rose with the increase in Dukes' stages (Table 4).

Table 4 Dukes' stages and local recurrence

Dukes' stage	Number	Local recurrence (%)
A	50	6 (12.00)
B	88	30 (34.09)[a]
C	75	37 (49.33)[a,b]
Total	213	73 (34.27)

[a]$P<0.01$, stage A *vs* stage B, stage A *vs* stage C, [b]$P < 0.05$, stage B *vs* stage C.

Sites of primary tumors and local recurrence

Tumors located in the middle segment of the rectum had a slightly higher rate of local recurrence than those in the upper segment of the rectum ($P<0.05$, Table 5).

Table 5 Primary tumor sites and local recurrence

Primary tumor sites	Number	Local recurrence (%)
Upper rectum	28	5 (17.86)
Middle rectum	52	21 (40.38)[a]
Lower rectum	133	47 (37.59)
Total	213	73 (34.27)

[a]$P<0.05$, *vs* middle rectum.

DISCUSSION

Local recurrence after curative surgery for rectal cancer is a major adverse prognostic indicator.

Although many investigations have been carried out in the prevention, early detection and treatment of this problem, about 7% - 65% of all rectal cancer patients still develop local recurrence[1-3]. In our series, the local recurrence rate was 34.27%. We also found that the causes are closely related to Dukes' stages, the length of distal rectum resected and the site of primary tumors, while the

pathological types and operational methods have no significant correlation with the postoperative recurrence.

Dukes' stages

Dukes' stage is an important factor related to postoperative local recurrence especially the pelvic recurrence. When tumors penetrate the whole rectal wall or metastasize to the regional lymph nodes (stage B and C) the local recurrence rate is 20%-40%. However, when these two negative factors combine together, the local recurrence rate will reach as high as 40%-60%[4,6]. In our series of 213 cases, the rate of postoperative local recurrence rose with the advancing stages, which clearly confirms the close correlation between local recurrence and extent of local invasion and regional lymph node involvement. From these observations there is a hope to reduce recurrence if extensive radical resections are routinely performed on patients with Dukes' B or C stage diseases, because these operations will further reduce the unseen residual tumors[7]. Other adjuvant treatments such as radiotherapy, chemotherapy or both, may be considered also for these high-risk patients.

Length of distal resection margin

The nature of transitional mucosa (the mucosa between the normal mucosa and the tumor) has been studied intensively. The transitional mucosa is a highly unstable precancerous lesion which closely links to postoperative recurrence and poor prognosis. The wider this region is, the shorter the post-operative five-year survival will be[8,9].

In clinical practice, when Dixon operation is performed, the length of proximal colon to be resected is seldom limited. However the length of the distal rectum to be resected is limited by several factors, including the preservation of sphincter functions, the available space of pelvic cavity and the operational manipulation. Preservation of sphincters will inevitably limit the length of distal resection margin. Moreover, the lower location of the tumor and the small pelvic cavity set a deep and narrow operation field with very limited exposure, which makes it extremely difficult for the surgeons to achieve a fairly clear distal margin. During the operation, the pulling and tracting will make the distal rectum to be resected seem longer. The intraoperative resection length sometimes is less than the resection length actually required.

The inadequate distal margin means increased chance of residual transitional mucosa or even occult residual cancer cells at the resection margin, which will eventually result in anastomotic recurrence. In general, a 3cm distal resection is required, while for highly malignant tumors, 5 to 7cm distal margin is necessary[10,11]. In our 86 cases of Dixon operation, 21 had anastomotic recurrence. The rates were 42.31% for those with a less than 3cm distal margin and 16.67% for those with a greater than 30cm distal margin.

Locations of primary tumors

The risk of local recurrence is directly correlated with the location of primary tumors. Cancers at the upper segment of the rectum behave like colon cancer. They are apt to metastasize distantly[2,3]. Because of their higher position, better exposure and easier operation manipulation, it is easy to carry out en bloc resection according to the principles of tumor surgery. Therefore, the rate of local recurrence is low. On the other hand, cancers at the middle and lower segments of the rectum are apt to locally recur because of the downward and lateral lymph drainage network and the lack of serosa to ward off local infiltration by the tumor. In these cases even extensive whole pelvic resection often cannot guarantee complete clearance. Therefore, the tendency to local recurrence is relatively high. And there are psychological causes too. For patients with low rectal cancers, Miles operation is the only possible curative treatment. But some of these patients refuse this proce dure due to various reasons. Instead they chose Dixon or Bacon operation, which often cannot ensure a true tumor free margin for their conditions. Local recurrence will be unavoidable in some of these patients.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):530-532

Comparison of long lasting therapeutic effects between succimer and penicillamine on hepatolenticular degeneration *

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Subject headings hepatolenticular degeneration/ drug therapy; succimer/therapeutic use; penicillamine/therapeutic use

Abstract

AIM To compare the long-term effect of succimer (Suc) with that of penicillamine (Pen) in treating hepatolenticular degeneration (HLD).

METHODS One hundred and twenty patients with HLD were divided into 2 groups. Group A (*n* =60) received Suc 750mg, po. bid. Group B (*n* =60) received Pen 250mg, po. qid. The period of maintenance treatment varied from 6 months to 3 years, averaging 1.5 years. Symptoms and therapeutic effects were evaluated by modified Goldstein scale.

RESULTS The total effectiveness of group A in two different periods of treatment were 80% and 85% respectively, higher than those of group B (58% and 59% respectively) (*P*<0.05). Suc also had obvious curative effects for the patients who failed in the use of Pen. There were fewer side effect in group A than in group B (*P*<0.05). Suc and Pen could increase urinary copper excretion effectively and continually.

CONCLUSION Suc is more effective and safer than Pen. Clinically, it can replace Pen as first-choice drug for long-term maintenance therapy of HLD.

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***Supported by the Natural Science Foundation of Anhui Province, No.** 97412001

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Received 1998-07-30

INTRODUCTION

Hepatolenticular degeneration (HLD) is an autosomal recessive disorder that causes changes in the basal ganglia and liver that respectively lead to neuropsychiatric disease, hepatitis and cirrhosis. The patients with HLD have to receive life long decoppering therapy, othewise the disease will run an invariably fatal course. Penicillamine (Pen) has remained the treatment of first choice for more than forty years because it is readily available and of proven efficacy in some patients. However, the use of this drug is associated with a wide range of toxic reactions and unsatisfactory curative effect for the patients with hepatic type[1]. We have used succimer (Suc) to treat HLD since 1990, with satisfactory results. The short-term therapeutic effect of Suc is much better than that of Pen[2]. But no study has been carried out to investigate the long-term curative effects of these two copper-binding agents. Our study is to further investigate the long-term therapeutic effects of Suc and Pen.

MATERIAL AND METHODS

Subjects

One hundred and twenty patients with HLD were chosen for this study. They were definitely diagnosed after Feb. 1994 and reexamined in our institute from Jan. 1996 to Dec. 1997. They were divided into group A (Suc therapy) and group B (Pen therapy). Based on clinical symptoms, they were classified as neurological type (including Wilson type and pseudosclerosis type) and hepatic type[3]. The severity of disease was graded from I to V according to the modified Goldstein method[4]. These two groups were comparable for their age, sex, course of disease, period of treatment, clinical classification and severity (Table 1).

Therapeutic methods

All patients had accomplished synthetucal copper-binding therapy with unithiol or EDTA before they started long-term maintenance treatment with Suc or Pen. The period of maintenance treatment varied

from 6 months to 3 years, averaging1.5 years. The patients of group A were given Suc that was produced by Shanghai Xinya Pharmaceutical Plant. Each pill contains 0.25g mese DMSA. Oral dosage was 20 mg/kg - 30 mg/kg body weight, and administered twice daily. The patients of group B were given Pen produced by Shanghai Xinyi Pharmaceutical Plant. Oral dosage was 20mg/kg-30mg/kg body weight and administered 4 times daily. Considering that both Suc and Pen could chelate other trace and macro-elements in the process of copper-binding, expecially zinc element, two hours after taking Suc or Pen, all patients of two groups were given 560mg zinc gluconate. Meanwhile, they were advised to take a copper-poor diet throughout the course of treatment and have their haemogram examined once a week. When patients were reexamined, their clinical conditions were independently judged by two experienced neurologists in our institute. Their haemogram, hepatorenal function, urinary trace and macro-elements were rechecked and compared with previous results. For the patients who did not obtain any effect from treatment, we asked to reexamine 2 months after they inter changed their copper-binding agents between group A and group B.

Therapeutic judgement criteria

Marked effectiveness Patient conditions were remarkably improved by two grades as compared with those before treatment.

Improvement Patient conditions were improved by one grade.

Ineffectiveness or exaceration Patient conditions had no obvious change or became worse.

Table 1 Characteristics of patients of group A and group B

Characteristics	Group A (n=60)	Group B (n=60)
Mean age (yr)	20±4.0	18±5.0
Males / Females	34 / 26	31 / 29
Mean course of illness (yr)	2.5±2.0	3.0±1.0
Length of treatment		
6mon to 2yrs	40	38
>2yrs	20	22
Clinical classification		
Wilson type	24	26
Pesudosclerosis type	16	15
Hepatic type	10	9
Modified Goldstein Scale		
Grade I	15	18
Grade II	29	27
Grade III	10	11
Grade IV	6	4

RESULTS

Clinical effects

We investigated the curative effects of Suc and Pen on HLD in different periods of treatment. The results showed that the total effectiveness of group A was 80% and 85% respectively, higher than those of group B (58% and 59%, P<0.05). Among 11 patients of group A who were demanded to replace Suc with Pen because of inefficiency of Suc, 1 (10%) was markedly improved and 2 (18%) improved, with an effectiveness rate of 27%. While in group B, 25 patients who changed to take Suc because of inefficiency of Pen, 5 (20%) were obviously improved, and 12 (48%) improved with an effectiveness rate of 68%. The difference was significant (χ^2=5.1, P<0.05, Table 2).

Table 2 Comparison of long-term curative effect of Suc and Pen

Group	Length of treatment	n	Markedly effective cases (%)	Improved cases (%)	Ineffective cases (%)	Total effective cases (%)
A	6mon to 2yrs	40	10(25)	22(55)	8(20)	32(80)[a]
	>2yrs	20	7(35)	10(50)	3(15)	17(85)[a]
B	6mon to 2yrs	38	5(15)	17(45)	16(42)	22(58)
	>2yrs	22	3(14)	10(45)	9(41)	13(59)

[a]P<0.05, compared with group B.

Table 3 Urinary trace and macro-elements changes after Suc and Pen therapy ($\bar{x}$±s)

Group	Time	n	Copper (μmol/L)	Zinc (μmol/L)	Calcium (mmol/L)
A	Before treatment	60	4.4±2.9	4.3±2.5	1.3±0.4
	After treatment	60	19±6.0[b]	21±7.3[b]	1.8±0.6
B	Before treatment	60	4.1±2.6	4.7±2.5	1.3±0.4
	After treatment	60	20±6.0[b]	20±6.9[b]	1.8±0.5

[b]P<0.01, compared with pre-treatment.

Side effects

During the period of maintenance treatment, 9 patients (15%) in group A suffered from gingival suffusion and nosebleeding, rash and mild abdominal distension; 22 patients (37%) in group B mainly manifested with high temperature, rash and cytoleukopenia. In both group A and B, there were 5 (5/9) and 16(16/22) patients who had the above side effects from 6 months to 2 years. These side effects could be abated or stoqped after the patients were given haemostatic, leukocyte-increasing drugs and antiallergics. No patient in both groups had to stop his treatment because of side effects. The incidence of side-effects in group A was notably lower than in group B, the difference being statistically significant (χ^2=7.36,P<0.01).

Laboratory studies

Long-term copper-binding treatment, remarkably increased the urinary copper and zinc excretion (P <0.01), indicating that both Suc and Pen could effectively facilitate urinary copper and zinc excretion (Table 3). In group B, the white blood counts of patients were decreased, but there was no statistical significance compared with the results of pre-treatment, and no significant changes in hepatorenal function either.

DISCUSSION

HLD is an autosomal recessive inheritant disease, abnormalities in serum ceruloplasmin, urinary copper excretion and copper accumulation in the liver have for many years suggested a primary defect in copper metabolism as the cause. By now, the gene responsible for HLD was located in choromosome 13q14.3, encoding a P-type ATPase. At least 70 different mutations have since been decribed in this copper-ATPase gene in individual with HLD[5]. Long-term anticopper therapy is necessary for patients to maintain their normal life. All neurologists considered Pen as a drug of first choice before Suc was clinically adopted. Suc is a new broad spectrum heavy metal antidote with low toxicity and high water solubility. It is easily discharged through urine after taken orally. We began to Suc to cure HLD in 1990 and found that it could improve effectively neurologic symptoms and facilitate evidently biliary copper excretion besides increasing clearly urinary copper excretion[6,7]. Comparing the long-term therapeutic effects between Suc and Pen on HLD, we found that Suc was Superior to Pen (P<0.05) because the former caused clinical symptoms to exacerbate less frequently than the latter did. Suc also had obvious curative effects for the patients who failed in Pen treatment. Side effects incidence of Suc was obviously lower than that of Pen ($P < 0.01$). Laboratory results showed that long-term use of Suc or Pen could increase effectively urinary copper and zinc excretion (P<0.01). As Suc has better short- and long-term therapeutic effects, less side effects and permenant urinary copper excretion function, we recommend that Suc should be used as a drug of first choice in long-term maintenance therapy of HLD.

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An experimental study in etiologic effect of pancreas divisum on chronic pancreatitis and its pathogenesis *

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Subject headings pancreatitis/etiology; pancreatitis/physiopathology; paucreas divism/physiopathology; paucreas divism/etiology; chronic diseases

Abstract

AIM To investigate the etiologic association of pancreas divisum (PD) with chronic pancreatitis and to clarify its pathogenesis.

METHODS A PD canine model was established in 32 dogs. The dogs were randomly divvided into 4 groups (n=8). Group I: The communicating branch between the dorsal and ventral pancreatic ducts was partly ligated Group IIa: The communicating branch was amputated and completely ligated Group IIb: The dorsal duct was amputated and ligated at 2mm distance to the minor papilla. Group III: A sham operation without any amputation or ligation was performed. Before and after operation, the activities of serum phospholipase A2 (PLA2) and amylase (Ams) were assayed and the basal pressures of the ducts were measured when secretin was injected. Pancreatic ductograhpy and the pathologic examination were made.

RESULYS The activities of serum PLA2 and ams in Group I,IIa, and IIb were sigificantly increased 5 - 80 days after operation. At sacrifice, the basal pressures of the ventral duct were significantly wiaher 30min-60min after provocation in Group I, IIa and IIb. The pressures of the dorsal duct were significantly increased in Group IIb but no difference in Group I and IIa. Under light microscopy the fibrosis of interlobus and periducts, the destruction of acini and infiltratiob of inflammatory cell in dorsal and ventral pancreas were found in Group IIb. But in Group I and IIa, this findings were pesent only in ventral pancreas. The electron microscopy showed that in ventral pancreas of Group I and IIa and the dorsal and ventral pancreas of Group IIb, the rough endoplasmic reticulum of the acinar cells showed granules-scaling, fusion and dilatation. The zymogen granules decreased and the mitochondria was swollen.

CONCLUSION PD is oue of etiologic factors in chronic pancreatitis. The pathogenesis is the functional obstruction of the minor papilla at the peak stage of secretion.

INTRODUCTION

The patients with pancreas divisum (PD) were considered to have a higher rish for chronic recurrent pancreatitis. But the ant the etiology and pathogenesis are still unclear. We established a canine model of PD in 32 dogs for investigating the etiologyic association and clarifying the pathogenesis.

MATERIALS AND METHODS

Animal model

Thirty-two healthy adult dogs of both sexes weighing about 10kg were used. Prior to the experiments, they were fasted for 24 hours. Under the sodium pentobarbital anesthesia, an abdominal midline incision was made and the head of pancreas was exposed. The partial ligation or amputation of the pancreatic duct was randomly made in 4 groups. Group I: the communicating branch between the dorsal and ventral pancreatic ducts was partly ligated at the middle segment ($n = 8$). Group IIa: the communicating branch was amputated and the remaining stump was ligated ($n = 8$) Group IIIb: the dorsal pancreatic duct was amputated and ligated at a 2- mm distance from the minor papilla ($n = 8$).

Pancreatic enxymes assay

The activities of serum phospholipase A2 (PLA2) and amylase (Ams) were assayed before ligation

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Received 1998-06-10

and 5, 10, 15, 30, 50, 80, 120, 150 and 180 days after operation by the method of radioimmunoassay[1] and blue starch respectively.

Manometry of pancreatic duct

The pressure of the dorsal and ventral pancreatic duct was measured before ligation and at sacrifice at 0, 15, 30, 60 and 90 min after itravenous injection of secretin (Kabi Vitrum, Stockholm, Sweden) at a dose of 1I U/kg. A polyvinyl catheter with a 0.8mm inner diameter and 1.0mm outer diameter was used. The probe contained an end orifice measuring 0.8mm in diameter and was filled with sterile saline. The perfusion took place at a constant rate of 0.25ml/min. The presure was recorded on a thermal pen recorder(Nippon Sanei 360, Tokyo, Japan).

Pancreatic ductography

Pancreatography was performed by retrofrade perfusion[2] via the major and minor papilla cannulation with 180mgI/ml Omnipaque (Nycomed Co, Norway) before operation and at sacrifice for investigating the pancreatic ductal changes.

Tissues preparation for light and electron minroscopy

The specimens for light microscopy were fixed with 10% formalin solution and embedded with paraffin wax. The sections were HE stained. Blocks of tissues ($1.0mm^3$) cut from pancreas and duodenal papilla for electron microscopy were fixed with 2.5% glutaraldehyde, postfixed with 1.0% OsO_4 dehydrated with ethanol, and embedded with Epon 812. The sections stained with uranyl acetate and lead citrate were examined under the Hitachi H-600 electron microscopy.

Statistical analysis

Results were wxpressed as mean±SD. Comparisons among groups were made using Scheffe's multiple test or Spearman's rank correlation analysis.

RESULTS

Biochemical assay

In Group IIb, the activities of the serum PLA2 and Ams were significantly increased after operation, especially during the postoperative 15-80 days. The activities in Groups I and Group IIa were slightly increased during the postoperative 5-15 days, but there was no statistically difference as compared with preoperation. There were no changes in the activities in Group III before and after operation.

Manometries of pancreatic duct

Before operation, the basal pressures of the dorsal and ventral duct were 0. 78 kPa ± 0.21 kPa and 0.69 kPa ± 0.24 kPa, respectively. When the secretin was administrated, the were slight increased at 30 and 60 min, but with no statistical difference (all $P>0.05$). During 60min-90min, the pressures rewailied at the preoperative levels.

At sacrifice

In ventral duct, the basal pressures in Groups I, II a, IIb and III were 0.80 kPa ± 0.3 kPa, 0.79 kPa ± 0.28 kPa, 0.64 kPa ± 0.20 kPa and 0.83 kPa ± 0.24 kPa, respectively. There was no significant difference as against preoperation. After secretin injection, the pressure at 30 min was significantly higher than that before operation in Groups I, IIa and IIb. At 60 min, the pressures in Groups I and IIa returned to the preoperational level at 90 min. In dorsal duct, the pressures were significantly increased in Group IIb ($P>0.001$). In Groups I, IIa and III, there were no significant differences before and after secretin injection.

Pathological changes

Light microscopy. In ventral pancreas, interlobus or/and periductal fibrosis, the destruction of acini and infiltration of inflammatory cell were found in Group IIb, slight periductal fibrosis in Groups I and IIa, and no abnormal histological changes in dorsal pancreas in Groups I and IIa, but chronic pancreatitis was present in Group IIb. The duodenal papilla was histologically normal.

Electron microscopy. The rough andoplasmic reticulim of the dorsal and ventral pancreatic acinar cells in Groups I,IIa and IIb showed granules-scaling, fusion and vacuolar dilatation. The Zymogen granules decreased and their electron density reduved. The mitochondria were swollen and the space around the nucleus was increased.

DISCUSSION

PD had been recognized by the 17th cantury anf it was found in approximately 7% of western autopsies. A higher incidence of chronic pancreatitis was discovered among the patients with PD, But their etiologic association has not been established, because so far there has been no animal models concerning PD. We established the animal model in 32 dogs through partial ligation or amputation of the communicating branch between the dorsal and ventral pancreatic duct. Not only the anatomic shapes but also the pathophysiologic bases were similar to that in human. PLA2 presents in the pancreas as an inactive zymogen, and inappropriate

intrapancreatic activation of PLA2 is thought to play an important role in the development of pancreatitis. In this study, the PLA2 activities in Groups IIa and IIb, especially in Group IIb were significantly increased, indicating the possibilities of pancreatitis. It was reported that PLA2 activities could be used to evaluate the severity of acute pancreatitis[3]. In Group IIb, due to all the pancreatic secretions occurring through the major papilla, not as the other groups, it was not surprising that more severe pancreatitis was induced.

The previous studies concerning the drainage of the pancreatic secretions were to measure the diameter of the duct by means of CT or ultrasound during secretin provocation[4,5]. It was not accurate because dilatation occurred in the pancreatic ducts when the periductal febrosis was obvious. In the present study, the pressures of the ventral duct in Group IIb at 30 and 60min were significantly higher than that before provocation. It implied the existance of physiologic obstruction. Chronic ventral and dorsal pancreatitis was found in Group IIb. But the similar changes were not seen in Groups I and III, and slight in Group IIa, and the duodenal papilla was histologically normal, confirming the association of pancreatitis with relative obstruction of secretion drainage. In Groups I and IIa, the communicating branch between the ventral and dorsal ducts were partly ligated or amputated. The juice secreted from the dorsal and ventral pancreas drained from the minor and major papilla, respectively. So, it is not likely to block the drainage. In Group IIb, all of the secretions drained via the major papilla. To bloch the drainage at the peak stage of the secretion was not avoided. Therefore, we have come to the conclusion. That there was clear etilogic association between PD and chronic pancreatitis.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):536-539

Preliminary study on the production of transgenic mice harboring hepatitis B virus X gene *

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Subject headings hepatitis B virus; gene, viral; transgenic animals; liver neoplasms; diseases models, animal

Abstract

AIM To establish transgenic mice lineage harboring hepatitis B virus X gene and to provide an efficient animal model for studying the exact role of the HBx gene in the process of hepatocarcinogenesis.

METHODS The HBx transgenic mice were produced by microinjecting the construct with X gene of HBV (subtype adr) DNA fragment into fertilzed eggs derived from inbred C57BL/6 strain; transgenic mice were identified by using Nested PCR; expression and phenotype of HBx gene were analyzed in liver from transgenic mic at the age of 8 weeks by RT-PCR, pathologic examination and periodic acid-schiff staining (PAS), respectively.

RESULTS Five hundred and fourteen fertilized eggs of C57 BL/6 mice were microinjected with recombinant retroviral DNA fragment, and 368 survival eggs injected were transferred to the oviducts of 18 pseudopregnant recipient mice, 8 of them became pregnant and gave birth to 20 F1 offspring. Of 20 offsprings, four males and two females carried the hybrid gene (HBx gene). Four male mice were determined as founder, named X1, X5, X9 and X15. These founders were back crossed to set up F1 generations with other ibred C57BL/6 mice or transgenic littermates, respectively. Transmission of HBx gene in F1 offspring of X1, X5 and X9 except in X15 followed Mendelian rules. The expression of HBx mRNA was detected in liver of F1 offspring from the founder mice (X1 and X9), which showed vacuolation lesion and glycogen positive foci.

CONCLUSION Transgenic mice harboring HBx gene were preliminarily established.

INTRODUCTION

Despite overwhelming epidemiological evidence linking persistent Hepatitis B Virus (HBV) infection and the development of hepatocellular carcinoma (HCC)[1], the pathogenetic mechanisms by which the virus contributes to liver cell transformation remain elusive. The case is mainly the limited host range and the lack of *in vitro* culture systems to propagate it. With the development of embryo microinjection technology, leading to the production of transgenic animals, it provided a powerful tool for studies of gene expression and creation of animal models of human diseases.

The HBx gene is one of the four genes in the HBV genome. It encodes a viral transactivator, the HBx antigen (HBxAg). This protein has been shown to convert immortalized mouse fetal hepatocytes into a fully malignant phenotype. Moreover, NIH 3T3 cells stably transfected with a HBx expression plasmid are carcinogenic in nude mice. Interestingly, recently it has been reported that the P53 and HBx proteins can be co-immunoprecipitated from HBV-related HCCs. Complexing of P53 protein by HBx protein should inhibit its DNA consensus binding and transcriptional transactivator function and provide a basis for the ways in which this interaction might contribute to malignant transformation[2].

These observations suggest. that HBx may play a part in the molecular pathogenesis of HCC in humans. In supprt of this hypothesis, it has been shown that high level expression og the HBx gene can lead to HCC in transgenic mice[3-5], although others have not observed the induction of HCC in independently derived *X* gene trasgenic mouse strains[6-8]. Noticeably, the mice that develop HCC

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were produced and maintained on a CD1 backround that displays a high spontaneous rate of HCC. This noght suggest that the HBx protein functions are a cofactor in the process of hepatocarcinogenesis, and that it may not be sufficient to induce HCC by itself[9].

To investigate the exact role of the HBx gene in the process of hepatocarcinogenesis. The HBx transgenic mice were produced by microinjecting the HBx gene of HBV (subtype adr) DNA fragment into fertilized eggs derived from inbred C57BL/6 strain mice that displays a lower spontaneous rate of HCC, and its phenotype were preliminarily analyzed by pathologic examination and PAS.

MATERIALS AND METHODS

Materials

Various restriction enzymes and other reagents. Modifying enzymes ECOR I, Hind III, BamHI, T4 DNA ligase, dNTP, Taq DNA polymerase, MMLV reverse transcriptase, etc, purchased from Promega, Sangon and Sigma Compant, respectively.

Mice. C57BL/6 mice (clean animal) were purchased from Animal Center. Shanghai.

Methods

Construction of recombinant plasmid. PSHDX42, a 6787bp recombinant retroviral vector plasmid containing the *X* gene of HBV (subtype adr) was constructed by DNA recombinant technique (Figure 1) (Plasmid constructs is to be published elsewhere).

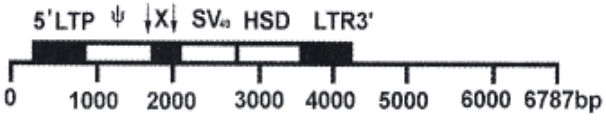

Figure 1 Structure of recombinant plasmid PSHDX42
X: HBx gene; ↓: BamHI

Production of HBx transgenic mice. Plasmid PXSHD42 was linearized with Ecor I and purified with QIAGEN. It was microinjected into the male pronucleus of the fertilized eggs of C57BL/6 mice. The injected eggs were then transplanted into the oviduct of pseudopregnany mice. Operated mice got pregnant and given birth.

Detection of transgenic integration. Genomic DNA was extracted from transgenic mice tail (about 1cm -1.5cm) at age of 2-3 weeks. Total DNA was extracted by the method described by Sambrook[10].

Nested-PCR. The primers were designed on human HBX (subtype adr) *X* gene sequence (synthesized by Cybersyn Biotechmology Company). The sequence of primer for the PCR were as follows:

Outer primer 1: 5'-CATGGCTGCTCGGGTGTGCT-3'
2: 5'-ATTAGGCAGACGTGAAAAG-3'
Inter primer 3: 5'-CTTTGTCTACGTCCCGTCGGCGCTGAATC-3'
4: 5'-CAGTCTTTGAAGTATGCCTCAAGGTCGGT-3

The PCR reaction I contained 5μ1 of 10×PCR Buffer, 3μ1 of 25mM Mg^{2+}, 3μ1 of 15mM dNTP, 1.5μ1 of 20pmol each of outer primer, 2U of Taq DNA polymerase, 1μg samle DNA. A 50μ1 total reaction volume was obtained by adding sterile water, the reaction procedure of PCR I was: beginning 5min at 95℃, then 30 cycles of 94℃ denature for 50 sec, ammealing at 55℃ for 1min and extension at 72℃ for lmin. The PCR reaction II contained 5μ1 of 10¡ÁPCR buffer, 3μ1 of 25mM Mg^{2+}, 3μ1 of 15mM dNTP, 3μ1 of 10pmol each of inter primer,2.5U of Taq DNA polymerase, 1μl-2μ1 PCR I products. The reaction procedure of PCR II was as follows: beginning 5 minat 94℃, 30 cycles of 94℃ denature for 50 sec, ammealing at 60℃ for 50 sec and extension at 72℃ for 1 min, and final extension at 72℃ for 10min. A 1.8% agarose gel was prepared and loaded with 10μ1 PCR II products, then electrophoresed at 45vol for 70min, and the result of electrophoresis was observed.

Detection of transgenic expreseeion. Tissue samples were removed from F1 transgenic mice liver at the age of 8 weeks and frozen in liquid nitrogen. Total RNA was extracted by the method described by Sambrook[10].

RT-PCR. The sequence of primers for RT-PCR were previously described as outer primer 1,2. First stranded cDNA was synthesized as follows: The RT reaction contained 1μg RNA,5μ1 of 10×PCR buffer, 3μ1 of 15 mM dNTP, 1μ1 of RNasin (80U/μ1), 2μ1 10pmol outer primer 1 or 2, 1μ1 MMLV (200U). A50μ1 total reaction volume was obtained by adding sterile DEPC treated water and RT was fouowed at 37℃ for DNA ploymerase, 3μ1 pf 10pmol outer primer 1,2 respectively. The reaction procedure of PCR was described as PCR I.

Histological procedures and cytohistochemical staining.

Liver tissues from F1 transgenic mice at the age of 8 weeks were divided into two portions: one portion fixed in 10% buffered formalin for Hematoxylin and Eosin as well as PAS stainin, the other quickly frozen in liquid nitrogen and used for RNA analysis.

RESULTS

Establishment of transgenic mice

Recombinant retrovial DNA fragment was microinjected into the male pronucleus of the 514 fertilized gees of C57-B1/6 mice, and 368 survived eggs were transferred to the oviducts of 18 pseudopregnant recipient mice, 8 of ther became pregnant and gave birth to 20 offspring mice. The zygotes survial rate and birth rate were 71% (368/514) and 5.4% (20/368). DNA extracted from tail of 20 offspring mice were screened by PCR amplification (Figure 2), and the results showed that four males and two frmales carried the hybrid gene (HBx gene). Four male mice were determined as founders, named X1, X5, X9 and X15. Total integration rate and efficiency of transgene was 30% and 1.1%, respectively.

Establishment of HBX transgenic mice lineage

The founders (X1,X5,X9 and X15) were back crossed to set up F1 offspring with other inbred C57BL/6 mice or transgenic littermates. Of 20 F1 mice from X1, 9 F1 mice carried the hybrid gene; of 18 F1 mice from X5, 8 F1 mice carried the hybrid gene; of 17 F1 mice from X9, 8 F1 mice carried the hybrid gene. Surprisingly, no mice integrated from F1 generation of X15 were detected by PCR.

Expression of HBx gene in F1 mice

We performed RT^{a2}PCR to examine whether HBx transgenes experess in liver tissues of F1 mice of transgenic mice lineages (X1,X5 and X9). The PCR products (439bp) were detected in the liver of F1 mice of transgenic mouse lineage (X1,X9) (Figure 3). NO Band was detected in the liver of F1 offspring of transgenic mouse lineage X5.

Phenitypic characteristics of transgenic F1 mouse

The liver from the F1 offspring of lineage (X1,X9) showed the presence of areas of altered hepatocytes which were made up of cells with pooly stained cytoplasm and PAS positive foci (Figure 4), but not in thir normal littermates.

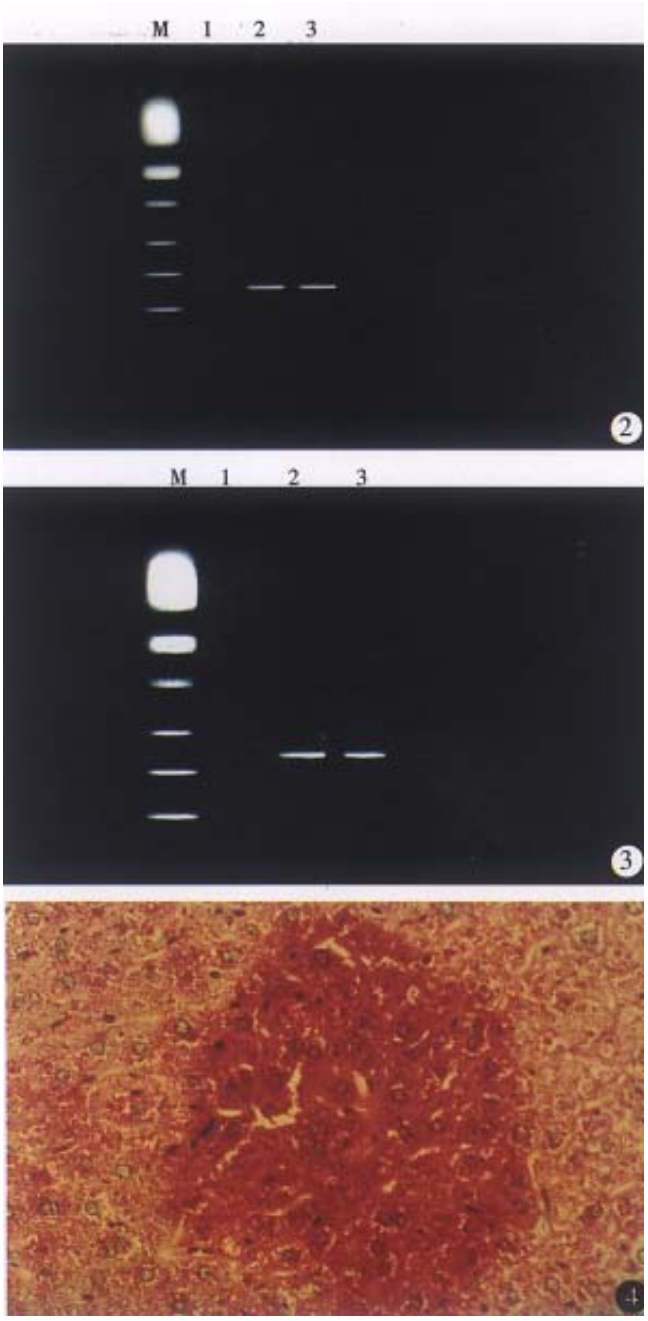

Figure 2 Electrophoresis analysis of Nested PCR products amplified from DNA obtained from tail tissue. lane 1, normal mouse (negative control; lane 2 and 3 (transgenic mice) showed positive bands (280bp). M, PCR marker (SABC).

Figure 3 Electrophoresis analysis of RT-PCR products. Lane 1, normal mouse liver tissue (negative control); lane 2 and 3 transgenic mice liver tissue showed positive bands (439bp). M, PCR standard marker (SABC).

Figure 4 Two-month old transgenic mouse liver showed glycogen positive foci PAS×200

DISCUSSION

Despite the increased frequency in the generation of transgenic animals, the methodologies for introducing exogenous DNA into mammalian early embryos have remained essentially unchanged since

the pioneering work of Gordon in the early 1980s[11]. To date, the most widely used method is microinjection of foreign DNA into the pronuclei of fertilized eggs. The method has enabled the production of transgenic animals from various mammalian species, but the success rate of generating a transgenic animal is extremely low. In this study, the zygotes survival rate and birth rate were 71% and 5.4% and efficiency of transgene was 1.1%. The lowas feeiciency of transgene may be influenced by many factors such as DNA structure, purity and concentration, gene vector and fertilized eggs as well as mouse strains, etc[12]. The result of F1 generation mice integration demonstrated that foreign gene can stably transmit to next generation in a normal Mendelian fashion. As for F1 generation from founder X5 low integration efficiency, it was thought that the founder X5 was likely to mosaics. The mechanism of DNA integration into mouse chromosomes following microinjection is unknown. One possibility is that spontaneous breaks occur in chromosome, possibly exacerbated by the microinjection technique and DNA repair endonuclease, and these breaks are sites for integration of linear DNA[12]

When the transgene results from random integration of a DNA fragment injected into fertilized eggs the pronucleus, its level of expression in the resulting transgenic animal varies and depends upon factors, such as the site, transgene cope number, genetic background of mouse, particularly promoter/enhancer of fusion gene. In this study, HBx gene under the control of retrovial promoter/ enhancer LTP and SV40 promoter, the expression of HBx gene was detected by RT-PCR in liver from partial transgenic mice lineage (X1, X9). The results demonstrated that the retrovial promoter/ enhancer LTR and SV40 promoter were probably functional, and HBX gene expression was influenced by different integrated site.

Above all initial histological examination of liver in HBX gene transgenic mice revealed the presence of patholoical change (vacuolation lesions) and glycogen positive foci. The results of phenotype observation were similar to KIM's[3], and were consistent with their preneoplastic lesions, and further identified HBx transgenic mice model established. To sum up, we have produced transgenic mice harboring HBx gene. This work has laid a solid foundation for further study on the function of JBx gene in hepatocarcinogenesis, successively analysis on these transgenic mice are being undertaking.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):540-543

Presence of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells *

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Subject headings liver neoplasms; BEL-7404; Fas gene; Bcl-2 proteins; gene expression; apoptosis

Abstract

AIM To study the expression of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells in order to analyze the possible relationship between cell growth regulation by alpha-fetoprotein(AFP) and Fas/Bcl-2 proteins.

METHODS BEL-7404 human hepatoma cells were maintained in RPMI 1640 medium supplemented with 10% new-born calf serum. Cells adhered to coverslips were used to detect Fas and Bcl-2 protein expression by the avidin-biotin complex (ABC) immunocytochemical assay.

RESULTS Immunocytochemical study showed that essentially all the BEL-7404 human hepatoma cells could express Fas and Bcl-2 proteins, although in various amount. No positive staining for Fas and Bcl-2 proteins was observed when cells were incubated with non-relevant sera, to establish the specificity.

CONCLUSION Fas apoptosis signals and Bcl-2 rescue/survival signals from apoptosis are expressed in BEL-7404 human hepatoma cells. The finding strongly implys that AFP-mediated cell apoptosis and growth enhancement are potentially associated with Fas and Bcl-2 proteins present in those cells.

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***Supported by National Postdoctor Science Foundation of China, No.** 199711.

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Reveived 1998-07-15

INTRODUCTION

Alpha-fetoprotein (AFP) is a major serum protein present in the early stages of development in mammals and other vertebrates, which virtually disappears in adult life. This protein is mainly synthesized by the fetal liver and the yolk sac. But, AFP may reappear in some tumors such as human primary hepatocellular carcinoma. It has also been found that AFP, like other serum proteins, can bind to specific membrane receptors on some cells such as macrophages, T lymphocytes, hepatoma cells, breast carcinoma cells and so on. AFP has immunoregulatory functions in a variety of experimental systems and some experimentally induced autoimmune diseases in animals can also be inhibited by the administration of AFP[1]. Our recent studies demonstrate that human AFP stimulates the proliferation of hepatoma cells *in vitro*[2]. Further investigation found that high concentrations of AFP resulted in hepatoma cell growth arrest[3], which suggests that AFP may be a biphasic cell growth regulator. However, the mechanisms of the growth-regulatory properties exhibited by AFP are largely unstudied.

Apoptosis routinely occurs during embryogenesis, histogenesis, metamorphosis, endocrine-dependent tissue atrophy and normal adult tissue turnover. Moreover, tumor regression is also often mediated through apoptosis as a result of X-irradiation and chemotherapeutic exposure in cancer cells[4,5]. The detection of apoptotic cells and apoptotic bodies is based on several well-established morphological features. These features include cell shrinkage, disconnection with neighboring cells, nuclear chromatin condensation, maintenance of cytoplasmic membrane integrity, strong eosinophilic cytoplasm and lack of an inflammatory reaction. Fragmentation of the cells leads to the appearance of membrane-bound apoptotic bodies. Apoptotic bodies are defined as small, roughly spherical or ovoid cytoplasmic fragments, some of which contain nuclear fragments. A previous report demonstrated that AFP and AFP-receptor antibody blocked the induction of apoptosis in HL-60 leukemia cells in culture[6]. But, high concentrations of AFP was found to induce apoptosis in human hepatoma cells[7]. These results have strong implications that growth-regulatory activity of AFP is, at least in

part, related to affecting apoptosis precess.

It has now been ascertained that cell apoptosis signals and rescue (survival) signals from apoptosis are mediated by a cell-surface transmembrane protein termed Fas and a cytosplasmic protein termed Bcl-2[8-10]. The human Fas protein (also designated APO-1) is a 48kDa cell surface glycoprotein that belongs to a family of receptors that includes CD 40, nerve growth factor receptors and tumor necrosis factor receptors. A series of studies indicate that apoptosis is mediated by the intercellular interactions of Fas with its ligand (Fas-L) or effectors. On the other hand, bcl-2 has been identified as an apoptosis inhibitor. The Bcl-2 protein (molecular mass 25kDa) is encoded by a gene involved in the *t* (14,18) chromosomal translocation and plays a central role in the inhibition of apoptosis. On the basis of Genbank identification, an amino acid sequence resembling a Fas-like peptide stretch has been detected in human AFP (39% identity, 23 amino acids in length). In a similar fashion, a sequence identifying with a Bcl-2-like amino acid stretch can also be discerned in human AFP (41% identity, 17 amino acids in length)[11]. Therefore, we speculate that Fas and Bcl-2 proteins in human hepatoma cells may contribute to the influence of AFP on apoptosis and growth regulation. We hereby investigated the expression of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells with avidin-biotin complex (ABC) immunocytochemical method.

MATERIALS AND METHODS

Cell lines and culture conditions

A human hepatoma cell line, BEL-7404, was maintained in RPMI 1640 medium (Gibco) supplemented with 10% new-born calf serum, at 37℃, 5% CO2 and 100% humidity. The RPMI 1640 medium was replaced with fresh medium every two to three days. For ABC assy, cells at a densitty of 5 $\times 10^3$ cells/mL were grown on coverslips, and adhensive cells were directly used. Cell viability was determined by mixing the cell suspension with 0.5% trypan blue (1:1).

Immunocytochemical assay

Antibodies used included a rabbit polyclonal antibody to Fas protein (Santa Cruz), and a mouse monclonal antibody to Bcl-2 protein (Dako). As described by the manusfacturers, the antibody against Fas protein is an affinity-purified antibody raised against a peptide corresponding to amino acids 260-279 mapping at the carboxy terminus of Fas protein of human origin. The specificity of the antiserum for Fas was confirmed by Western blotting and immunohistochemistry. The antibody against Bcl-2 protein belonged to mouse IgG1 subclass. The antibody reacted specifically with Bcl-2 protein as demonstrated by immunoblotting and immunoprecipitation.

The monoclonal and polyclonal antibody reactivities were visualized using Vectastain ABC kit (Vector) following the vendor's instructions with a few modifications[12]. Briefly, adhensive cells were fixed in 4% paraformaldehyde for 5min-8min at room temperature, then treated with 0.05M Tris-buffered saline (TBS, pH 7.2) containing 0.4% Triton X-100 for 15min at room temperature. The coverslips were then incubated with 1% bovine serum albumin in TBS containing 0.4% Triton X-100 for 30min at 37℃. For the detection of Fas protein, prior to incubation with antibodies, endogenous peroxidase activity was irreversily inhibited by treatment with 5% hydrogen peroxide in methanol solution at room temperature for 30min. After washing in TBS, primary antibodies to Fas were applied for 1h at 37℃. After washing 3 times, coverslips were incubated with biotinylated sheep anti-rabbit secondary antibodies at 37℃ for 45min followed by avidin biotin peroxidase complex at 37℃ for 45min. For color development, a peroxidase substrate solution, 0.05% 3,3'-diaminobenzidine (Sigma) in TBS containing 0.01% hydrogen peroxide, was applied for 5min-10min at 37℃. For the assay of Bcl-2 protein, primary antibodies for Bcl-2 were reacted with the cells for 1h at 37℃. After incubation with the biotinylated secondary antibodies (horse anti-mouse antibodies) for 45min at 37℃, the coverslips were treated with alkaline-phosphatase-conjugated avidin-biotin complex for 1h at 37℃. Development reagents contained 33μl of nitroblue tetrazolium salt (75mg/mL in 70% dimethylformamide) and 25μl of 5-bromo-4-chloro-3-indocyl phosphate (50mg/mL in dimethylformamide) in 7.5mL TBS. For color development, cells were incubated in the color solution for up to 4h at 37℃ in the dark. The coverslips were finally rinsed in water. The primary antibodies were replaced by control non-relevant sera to monitor specificity of ABC immunocytochemical staining.

RESULTS

On the basis of preliminary experiments, rabbit anti-human Fas antibody was used at a dilution of 1:80, sheep anti-rabbit IgG at 1:50, and rabbit ABC at 1:100, respectively. The dilution of mouse anti-human antibodies against Bcl-2 was 1:100. Horse anti-mouse IgG and mouse ABC were used at 1:80 and 1:100, respectively. The use of appropriately diluted reagents minimized troublesome nonspecific background staining.

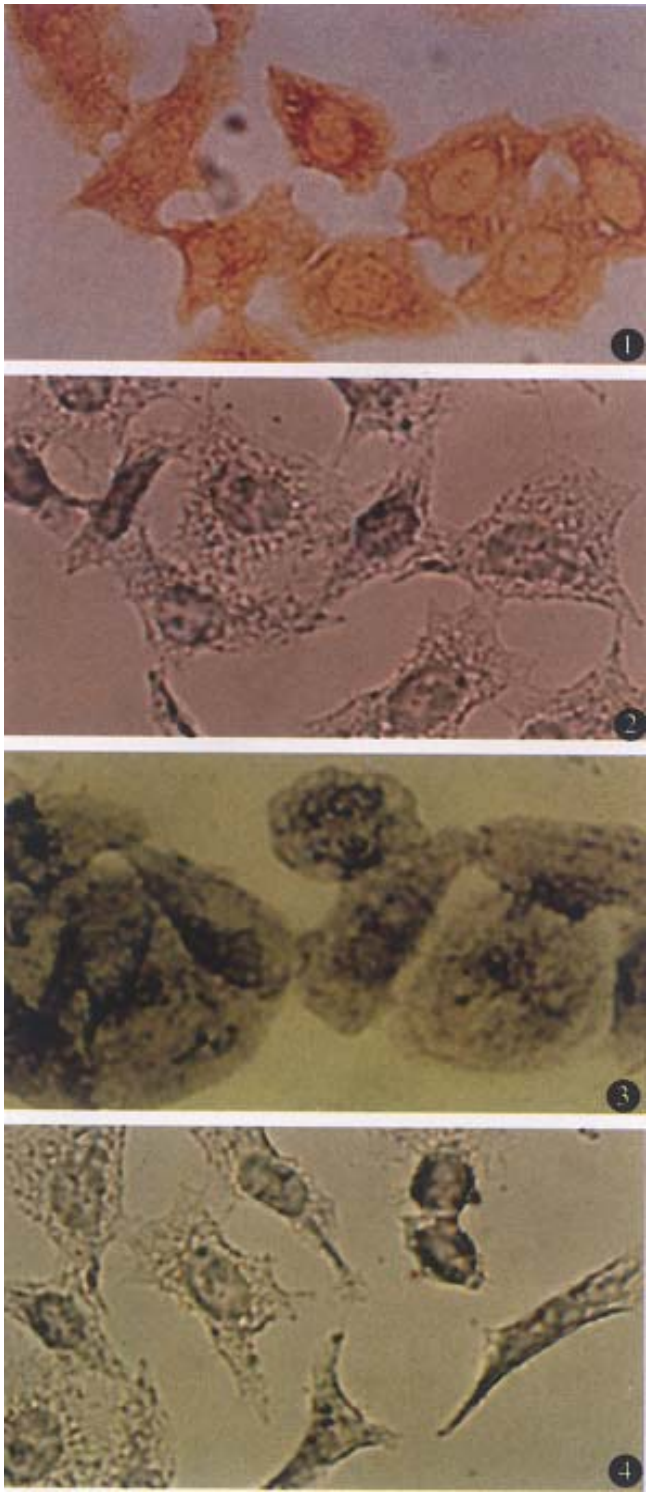

Figure Detection of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells. (1) Fas antigen was stained by the avidin- biotin-peroxidase complex. Reaction products were visible in almost all the cells. (2) Same as in 1, except that rabbit anti-human polyclonal antibody to Fas was replaced by normal rabbit serum. (3) BEL-7404 cells were incubated with mouse anti-human monoclonal antibody to Bcl-2. Positive grains detected by alkaline-phosphatase-conjugated avidin-biotin complex were also visible in almost all the cells. (4) Same as in 3, except that the primary antibody to Bcl-2 was replaced by normal mouse serum. Original magnification×200.

Figure 1 shows ABC detection of Fas antigen in BEL-7404 cells. When primary antibodies to Fas were incubated with BEL-7404 cells, large numbers of brown grains were detected on the membrane of essentially all the cells, although the grain distribution varied. Treatment of the cells with normal rabbit serum resulted in a marked reduction in the number of specific grains (Figure 2). When the primary antibodies against Bcl-2 were used, purple grains were present diffusely throughout the cytoplasm of BEL-7404 cells with sparing of the nuclei. The number of grains in the cytoplasm varied, but essentially all the cells, including those undergoing mitotic division, were considered to contain Bcl-2 protein (Figure 3). However, when cells were incubated with normal mouse serum under the same experimental condition, no accumulation of grains in the cells was observed (Figure 4).

Overdevelopment of the color reaction can produce a high level of non-specific staiing, but this was significantly reduced by careful monitoring of the reaction after the addition of color reagents.

DISCUSSION

For study of gene expression, immunocytochemical staining can be used to detect final products of gene expression and their location in cell, though it cannot be used to detect directly genes. In the present study, Fas and Bcl-2 proteins have been detected by ABC immunocytochemical method in cultured BEL-7404 human hepatoma cells. We consider that ABC assay is rapid, reliable, sensitive and economical to use, and there are no known health hazards associated with the reagents used, which provides a very useful tool for investigating the gene expression[12].

Some studies demonstrate that cell apoptosis is mediated by the intercellular interactions of Fas with its ligand or effectors, and Bcl-2 protein can inhibit apoptosis[8-10]. As mentioned in the "Introduction", amino acid sequences of Fas and Bcl-2 proteins can be detected on some domains of human AFP[11]. Moreover, Bcl-2 site is localized adjacent to a proposed hinge region of human AFP molecule, which allows rotational flexibility. A conformational change in the tertiary structure of AFP, possibly induced by excessive ligand binding, can expose such a Bcl-2 site, which is normally hidden in a molecular crevice. According to the present result, taken together with previous published data, we speculate that dimerization or binding of low concentrations of AFP with its normal molecular configuration to Fas protein could blunt the apoptosis signals, resulting in enhancement of cell growth. On the other side,

dimerization or binding of high concentrations of AFP with possible conformational change to the exposed Bcl-2 signal site could block the rescue/survival signals, causing the induction of apoptosis. In this fashion, AFP might function in both the up and down-regulation of cell growth by employing a binding or dimerizing mechanism to apoptotic mediators.

The oncoproteins are products of the proto-oncogenes, which now include myc, myb, fos, jun, ski, ets, cbl, erb A and possibly many others. Most oncoproteins have turned out to be transcription factors, which function as molecular switches that sense incoming signals and modulate the transcription of specific genes. Many oncoproteins have functional partners with which they heterodimerize to bind DNA, such as fos and jun, myc and max, etc. Protein-protein and protein-DNA interactions of the oncoproteins are often mediated through helix-loop-helix and leucine zipper motifs. Tumor suppressor proteins (*p*53 and retinoblastoma protein) can function as growth suppressors. It has been found that AFP gene promoter can be regulated by some transcription factors such as AP-1[13], although the activation of the oncogenes c-fos, c-jun and c-myc might not directly *in volve* the basal level of AFP gene expression in hepatoma cell lines[14]. The mutation of p53 gene occurred in the early stage of hepatocarcinogenesis may be correlated with the initiation of hepatocarcinogenesis, and mutant P53 protein probably related to the reactivation of AFP gene[15]. On the other hand, Genbank computer-generated sequence identities detected on human AFP domains demonstrated that amino acid identities for the oncogenes c-erb A, c-myc, rel, myb, ras ranged from 29% to 54% over lengths from 13 to 24 amino acid, largely in human AFP domains 1 and 2. In comparison, the tumor-suppressor (retinoblastoma, Rb) protein identities appear to reside only on domain 1 of human AFP[11]. Thus, it is possible that the growth-regulatory activity of AFP may also be mediated by some oncoproteins and/or tumor suppressor proteins. The exact relationship between AFP growth regulation and oncoproteins as well as tumor suppressor proteins still needs to be further investigated in the future.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):544-547

Combined Traditional and Modern Medicine

A comparison between Zhishi Xiaopiwan and cisapride in treatment of functional dyspepsia *

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Subject headings Dyspepsia/therapy; Zhishi xiaopi; cisapride; gastric emptying

Abstract

AIM To compare the therapeutic effect of the herbal medicine Zhishi Xiaopi with that of Cisapride in the treatment of functional dyspepsia (FD).

METHODS Fifty-one FD patients were randomized into Herbal group (*n* = 27) and Cisapride group (*n* = 24). Two two groups were given a four-week treatment of *Zhishi Xiaopiwan* 100ml, tid, a.c. and Cisapride 5mg, tid, a.c. respectively. Patients' symptoms were assessed and 39 patients' (22 of Herbal group and 17 of Cisapride group) gastric liquid emptying times were measured with ultrasonography before and after the treatment.

RESULTS The therapeutic effective rates of Herbal group and Cisapride group were 81.49% and 87.50% (*P*>0.05). The half gastric emptying time (GET_{50}) and gastric emptying time (GET) of healthy controls and FD patients were 36.12min±10.22min *vs* 52.95min±13.49min and 87.07min±21.11min *vs* 120.74min±23.08min (*P* <0.001). The GET50 and GET of Herbal group before and after the treatment were 51.63min± 13.15min *vs* 45.62min±10.82min and 117.34min ± 23.29min *vs* 103.26min±22.19min (*P*<0.01). The results of Cisapride group were 54.66min± 14.14min *vs* 40.95min±11.29min and 125.12min ±24.47min *vs* 95.49min±22.31min (*P*<0.01). The differences in values (median) of GET_{50} and GET for Herbal group and Cisapride group before and after treatment were 5.75min *vs* 17.18min and 13.22min *vs* 33.54min (*P*<0.05).

CONCLUSION Delayed gastric emptying is one of the pathogenesis of FD. Both *Zhishi Xiaopi* pills and *Cisapride* can effectively alleviate the symptoms of FD and accelerate gastric liquid emptying. The effect of Zhishi Xiaopiwan on enhancing gastric motility is comparable with but less than that of Cisapride.

INTRODUCTION

Dyspepsia is a common syndrome, Outpatients in gastrointestinal clinics complaining of dyspeptic symptoms amount to about 30%-40% of the total visits. Among them, more than half have no organic lesions after examination. So it is called functional dyspepsia (FD). Some of the patients with FD present symptoms suggestive of delayed gastric emptying. So gastrointestinal hypomotility is considered one of the pathogenesis. In this study, we compared the therapeutic effect of Zhishi Xiaopiwan made of (herbal medicine) with Cisapride in the treatment of FD by assessing symptomatic improvement and measurement of gastric liquid emptying time.

MATERIALS AND METHODS

Diagnosis of functional Dyspepsia

Epigastric pain, discomfort or bloating, postprandial fullness, eructation, nausea and other upper abdominal symptoms lasting at least for 4 weeks; esophagitis, peptic ulcer, upper gastrointestinal erosion and neoplasm were excluded by endoscopy; diseases of liver, gallbladder, pancreas and lower gastrointestinal tract by laboratory, ultrasound and X-ray examinations; and diabetes, hyperthyroidism, connective tissue diseases and history of abdominal surgery were excluded.

Patients and healthy controls

Fifty-one patients meeting the above diagnostic criteria were randomly divided into two groups:

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Received 1998-11-09

Herbal group, 27 patients (average age, 45.1±9.67 year; range, 25-63 years; a ratio of men/women, 10±17) and Cisapride group, 24 patients (aveaage age, 48.5±12.9 years; range, 26 - 72 year; a ratio of men/women, 13:11). Healthy control group consisted of 10 volunteers without gastrointestinal symptoms (average age, 41.4±11.75 years; range, 25-58 year; a ratio of men/women, 7:3).

Symptom assessment

Five symptoms (epigastric pain or discomfort, bloating, postprandial fullness, eructation and nausea) were assessed for each patient once a week or two weeks. Symptoms were assessed according to Stanghellini criteria[1]: 0 = absent, 1 = occasionally present and not affecting patients¡ä daily activities; 2 =present moderately often, slightly affecting their activities; 3 = present moderately or more often, affecting considerably patients' activities. The symptom complex index (SCI) was calculated by dividing the summation of all presenting symptoms' scores with the number of symptoms.

Therapeutic efficacy assessment criteria

Noticeable efficiency (NE): SCI was less than pre-treatment value by 2 or more; efficiency: SCI was less than pre[a2]treatment value by 1 or more but less than 2; and inefficiency: SCI was less than pre-treatment value by less than 1.

Measurement of gastric liquid emptying

Gastric liquid emptying was measured in one vertical section of antrum with real time ultrasonography as used by Marizo[2]. All medication was stopped for 3 days and the patients were fasted for 12 hours before the test. Milk of 250ml with a total caloric value of 597KJ, prewarmed to 37℃, was used as a testing meal.

Treatment

Herbal group was treated with Zhishi Xiaopiwan in the form of decoction (100ml three times a day before meal) for 4 weeks. Zhishi Xiaopiwan consisted for the following ingredients:*Citrus aurantium L*.15g, *Officinal Magnolia Bark* 12g, *Tangshen Asiabell Root* 10g, *Largehead Atractylodes Rhizome* 10g, *Poria* 12g, *Ternate Pinellia Tuber* 10g, *Golden Thread Rhizome* 3g, *Nardostachys Rhizome and Root* 6g, etc. If pain was severe, *Paniclate Swallowwort Root* 10g and *Corydalis yanhusuo W.T.Wang* 15g were added. If bloating was severe, *Finger Citron* 10g, *Akebi Fruit* 10g and *Costus Root* 10g were supplemented. If eructation and nausea were severe, *Inula Flower* 10g and *Bamboo Shavings* 5 g were added. If there was regurgitation, *Ark Shell* 30g and *Cuttle Bone* 30g were used Cisapride group was treated with Cisapride (5mg three times a day before meals) for 4 weeks.

Statistical analysis

Student's *t* test, χ^2 test, rank sum test and linear regression were used and *P* values less than 0.05 were considered significant.

RESULTS

Therapeutic efficiency of symptoms

Both Zhishi Xiaopiwan and Cisapride could effectively alleviate patients' symptoms (Tables 1-3). There were no significant difference between the efficencies of the two medications on the symptoms of the FD, and in improvement SCI between two groups after treatment. The total efficacy rates (NE +E) of Herbal group and Cisapride group were 81.49% and 87.5% respecitively ($P > 0.05$). During treatment, 3 patients of Cisapride group had fewer gastrointestinal symptoms (1 with lower abdominal pain, 2 with loose stool). Patient in Herbal group had no side reactions, but with no statistical significance ($\chi^2 = 3.59, P > 0.05$).

Table 1 Comparision of the theraputic efficiency for symptoms relief

Symptoms	Group	Cases	NE[a](n)	E[a](n)	IE[a](n)	EF[a](%)
Epigastic pain	Herbal	16	1	10	5	68.75[b]
	Cisapride	15	4	7	4	73.33
Bloating	Herbal	22	10	8	4	81.89[b]
	Cisapride	18	13	3	2	88.89
Postprandial fullness	Herbal	19	9	8	2	89.47[b]
	Cisapride	16	12	3	1	93.00
Eructation	Herbal	14	5	7	2	85.71[b]
	Cisapride	17	10	4	3	82.35
Nausea	Herbal	6	3	3	0	100.00[b]
	Cisapride	8	2	4	2	75.00

[a]NE: noticeable efficiency, E: efficiency, IE: inefficiency, ER: efficiency rate; [b]*P*>0.05 *v* Cisapride group.

Table 2 Comparision of symptoms complex index

Group	Cases	Before treatment	After treatment	Differential values
Herbal	27	2.28±0.32	1.02±0.60[a]	1.26±0.56[b]
Cispride	24	2.19±0.40	0.71±0.55[a]	1.49±0.56

[a]*P*<0.001 *v* before treatment, [b]*P*>0.05 *v* Cisapride group.

Table 3 Comparision of the clinical theraputic efficiency rate

Group	Cases	NE[a] rate %(n)	Ea rate %(n)	IEa rate %(n)
Herbal	27	25.93 (7)[b]	55.56 (15)[b]	18.51 (5)[b]
Cisapride	24	37.50 (9)	50.00 (12)	12.50 (3)

aNE: noticeable efficiency, E: efficiency, IE: inefficiency; [b]*P* > 0.05 *v* Cisapride group.

Gastric liquid emptying

Gastric liquid emptying tests were done in 10 healthy volunteers and 39 FD patients. Half gastric emptying time (GET_{-50}) and total gastric emptying time (GET) of health controls were 36.12 min ± 10.22min and 87.07 min ± 21.11 min. Those of FD patients were 52.95 min ± 13.49 min and 120.74 min ±23.80min, both of which were longer than those of healthy controls ($P < 0.001$). If the normal GET_{50} range was set as from 16.09 to 56.15 (mean ±1.96 SD), there were 14 (35.90%) patients with delay of gastric emptying.

Both Zhishi Xiaopiwan and Cisapride could shorten the gastric liquid emptying time (Table 4). After treatment, there was still significant difference between the gastric emptying time of Herbal group and control group ($P < 0.05$), but there was no significant difference between those of Cisapride group and control group ($P>0.05$). The median difference of GET_{50} and GET of Herbal group before and after treatment were 5.75 min and 13.22mine respecitively, and those of Cisapride group were 17.18 min and 33.54 min ($0.25<P<0.05$). So the effect of Zhishi Xiaopiwan in enhancing gastric emptying is less than that of Cisapride.

Table 4 Comparision of gastric emptying time between before and after treatment

Group		Cases	GET50 (min)	GET (min)
Control		10	36.12±10.22	87.07±21.11
Herbal	Before treatment	22	51.63±13.15[a]	117.34±23.29[a]
	After treatment	22	45.62±10.82[b,c]	103.26±22.19[b,c]
Cisapride	Before treatment	17	54.66±14.14[a]	125.01±24.47[a]
	After treatment	17	40.95±11.29[b,d]	95.49±22.31[b,d]

[a]$P < 0.01$ *v* control group, [b]$P < 0.01$ *v* before treatment, [c]$P < 0.05$ *v* control group, [d]$P>0.05$ *vs* Control group.

DISCUSSION

In this study, we found 35.9% of FD patients had delayed gastric liquid emptying, which is similiar to the results of 23%-55% reported previously[3,4]. This indicates that quiet a few FD patients have gastric hypomotility. Gastrointestinal manometric techniques[1,5] showed that during fasting, the cycles of Migrating Motor Complex (MMC) of FD patients are less than those of healthy subjects. No matter during fasting or digestive period, the amplitude and frequency of the contractions of antrum and duodenum of FD patients are all less than those of healthy subjects. During digestive period, the numbers of duodenal propulsive peristalsis and the coordinating contractions between antrum and duodenum are less than those of healthy subjects. Ultrasonographic techniques[6] also showed that the postprandial antrum contractions of FD patients are incomplete with small waves of irregular rhythm, as compared to the complete, even and rhythmical peristalsis of healthy subjects. The gastrointestinal hypomotility and the incoordination between antrum and duodenum may induce delay of gastric emptying. As an agonist of $5\text{-}HT_4$ receptor, Cisapride can act on the receptors of the intermediate and terminal neurons of myenteric nerve plexus in gastrointestinal smooth muscle and improve the gastrointestinal motility by promoting cholinergic nerves to release acetylcholine. It can increase the amplitude and freqauency of the gastric contractions and the numbers of coordinating contractions between antrum and duodenum to accelerate gastric emptying.

Symptomatology of FD is very similar to that of "Piman Zheng", a name of disease in Traditional Chinese medicine with bloating as the chief complaint. Zhishi Xiaopiwan is the commonly used Chinese medicine for "Piman Zheng". So we chose this medicine to treat FD and compared it with Cisapride. The results showed that Zhishi Xiaopiwan could effectively ameliorate the symptoms of FD, and its total efficiency rate (81.49%) was not significantly different from that of Cisapride (87.5%). Gastric emptying examinations indicated that Zhishi Xiaopiwan also could accelerate the gastric liquid emptying, although its effect was less than that of Cisapride. Its effect on enhancing gastrointestinal motility was proven in previous animal exeriments. The main ingredient of Zhishi Xiaopiwan-*Citrus aurantium L.* could prolong the cavine intestinal active duration of MMC by reducing the duration of the phase I and prolonging the duration of the phase II[7]. Other ingredients, such as *Tangshen Asiabel Root, Rhizome Atracty lodes macrocephalae, Ternate Pinellia Tuber* and *Golden Thread Rhizome*, could increase the frequency of rat gastric electric spikes and amplitude of gastric contractions and regulate the disturbance of gastric electric rhythm. *Rhizome Atractylodes* macrocephalae might act on cholinergic receptors to activate the gastric movement[8,9].

Not all FD patients had delayed gastric emptying. This indicates that gastrointestinal hypomotility is not the sole pathogensis of FD, other factors such as hypersensitiveness of gastric mucosa, disturbance of gastric accomodation and failure of gallbladder contraction might also be involved in the pathogenesis[10]. The inconsistency between the symptomatic relief and improvement of gastric emptying by Zhishi Xiaopian also suggests

that the action of the herbal medicine to improve symptoms might be through mechanisms other than promoting gastric motility, which shoujld be further studied in the future.

In conclusion, we found that delay of gastric emptying was one of the pathogenetic factors of FD, and both Zhishi Xiaopiwan and Cisapride could ameliorate the symptoms of FD and accelerate the gastric liquid emptying in certain percentage of patients. In this aspect, Cisapride is better than the herbal medicine we used.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):548-549

Effects of Fuzhenghuayu decoction on collagen synthesis of cultured hepatic stellate cells, hepatocytes and fibroblasts in rats *

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Subject headings Fuzhenghuayu decoction; collagen synthesis; hepatic stellate cells; hepatocytes; fibroblasts

Abstract

AIM To study the mechanism of Fuzhenghuayu (FZHY) decoction on anti-liver fibrosis.

METHODS FZHY 10% decoction sera was incubated with rat normal subcultured hepatic stellate cells (HSC) and fibrotic primarily cultured HSC, normal and fibrotic hepatocytes and subcultured skin fibroblasts seperately. Cell intracellular and extracellular collagen synthesis rates were measured by the method of [^{3}H] Proline impulse and collagenase digestion.

RESULTS For primarily cultured HSC and hepatocytes, both of intracellular and extracellular collagen synthesis rates decreased in the drug sera group. For the normal subcultured HSC and primarily cultured hepatocytes, the extracellular collagen secretion was decreased obviously by the drug sera, and intracellular collagen synthesis rates were inhibited to some extents. For fibroblasts, both intracellular and extracellular collagen synthesis rates were inhibited somewhat, but no significant differences were found.

CONCLUSION The mechanism of FZHY decoction on anti-liver fibrosis may be associated with inhibition of liver collagen production.

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*Project supported by the National Natural Science Foundation of China, No. 39570889

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Received 1998-07-16

INTRODUCTION

Liver fibrosis is the common pathological feature of chronic liver diseases, and is closely associated with changes of liver cell functions. In order to investigate the mechanisms of Fuzhenghuayu decoction action on liver fibrosis, the drug serum was collected and incubated with cultured rat hepatic stellate cells (HSC), hepatocytes and fibroblasts, and then cellular functions were observed.

MATERIALS AND METHODS

Animals

Wistar male rats were purchased from Shanghai Animal Center, Chinese Academy of Sciences, among them, rats weighing 180g-200g rats were used for isolations of hepatocytes, 350g-450g rats were used for isolation of HSC. SD rats, pregnant for 12-14 days were the gifts of Shanghai Institute of Family Planning and used for skin fibroblast isolation and culture. All rats were maintained with food and water available *ad labium*.

Reagents

Minimum essential medium Eagle (MEM), 199 Medium (M199) and Dubocal modified Eagle's Medium (DMEM) were purchased from GIBCO, USA. Pronase E, type IV collangenase, Metrizimide, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) from Sigma Co., USA. And L-[5-^{3}H] proline ([^{3}H]Pro) from Amersham Co., England.

Drug

Fuzhenghuayu (FZHY) decoction consists of *Cerdeceps, Semen Persiciae, Radix Salviae* Miltiorrhizae, etc. Shanghai Zhonghua Pharmaceutical Factory made the decoction into a kind of fluid extract. Each gram of the fluid extract contained 2703g of the above raw herbs.

Cell isolation and culture HSC isolation and culture were performed according to the modified Freidman method[1], and hepatocyte isolation according to the modified method[2]. Fibroblast followed E Zheng's method[3].

Model establishment[4] Male Wistar rats received 0.5% DMN dissolved in 0.15mol/L NaCl, at a dose of 10μl of DMN/100kg i.p., for 3 consecutive days each week for 3 weeks. The pair fed controls

received an equivalent amount of saline.

Drug sera were prepared Using our own method[5].

Grouping and drug sear incubation The cultured cells were divided into control and drug serum groups. The control was incubated with 199 medium containing 10% normal rat serum, and the drug group was incubated with 199 medium containing 10% drug serum, for 72h in HSC and fibroblast cells, for 48h in hepatocytes and 24h for fibrotic HSC.

Assay of collagen synthesis rates Greets method was used[7].

Statistics Two tails student t test was used for statistical analysis.

RESULTS

Effects on HSC collagen synthesis

The drug sera markedly inhibited the intracellular collagen synthesis in normal subcultured HSC, and both intracellular and extracellular collagen productions in fibrotic HSC.

Table 1 Effects of drug sera on HSC collagen synthesis rate (n = 4, % $\bar{x}$±s)

Group	Normal subcultured HSC		Fibrotic HSC	
	Intracellular	Extracellular	Intracellular	Extracellular
Drug sera	0.31±0.21	2.70±0.10[a]	0.12±0.09[a]	0.80±0.34[b]
Control	0.57±0.37	4.15±0.95	0.33±0.10	1.72±0.53

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

Effects on hepatocyte collagen synthesis

The drug sera could inhibit extracellular collagen synthesis more obviously in fibrotic hepatocytes, than in the normal cells. The drug sera could also inhibit intracellular collagen synthesis of fibrotic hepatocytes (Table 2).

Table 2 Effects of drug sera on hepatocyte collagen synthesis rate (n = 4, % $\bar{x}$±s)

Group	Normal subcultured HSC		Fibrotic HSC	
	Intracellular	Extracellular	Intracellular	Extracellular
Drug sera	0.34±0.05	0.23±0.04[a]	0.84±0.16[a]	0.43±0.12[b]
Control	0.31±0.09	0.30±0.02	1.24±0.50	0.60±0.14

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

Effects on NIH/3T3 fibroblast collagen synthesis

The drug sera could inhibit both intracellular and extracellular collagen synthesis to some extent, but no significant difference was found (Table 3).

Table 3 Effects of drug sera on NIH/3T3 fibroblast collagen synthesis rate (n = 4, % $\bar{x}$±s)

Group	Intracellular	Extracellular
Drug sera	1.53±0.50 (6)	8.89±3.66 (6)
Control	2.03±0.75 (5)	12.62±1.03 (4)

In bracket was the case numbers.

DISCUSSION

Collagens are the main components of extracellular matrix, which play an important role in keeping liver structure and functions. If collagen production increased or decomposition decreased, its metabolism would break and lead to liver fibrosis. It was found that HSC could transform to myofibroblasts under stimulation of cytokines induced by hepatocyte injury, and was the key cell for synthesis and secretion of collagen in liver. In the paper, both normal subcultured HSC and fibrotic HSC showed significance in collagen production. Although hepatocyte has the function of collagen production, which is low in normal hepatocytes, and increased obviously in fibrotic hepatocytes. This was also observed in our study. Fibroblasts had many subtypes, all of which could produce extracellular matrix, and were used for cell models in investigating liver fibrosis instead of HSC[8,9]. In this study, rat skin fibroblast showed ability of collagen production.

Besides anti-etiology therapy, regulation of collagen metabolism, including inhibition of collagen synthesis and increase of collagen decomposition, could protect or delay the formation of liver fibrosis, while inhibition of collagen production in liver is one of key steps for anti-liver fibrosis. In our previous clinical and animal studies, Fuzhenghuayu decoction showed good effects on liver fibrosis[10-13]. In the present study, serum was collected from rats fed on Fuzhenghuayu decoction by seropharmacological method and incubated with 3 kinds of cells. The results showed that the drug sera could decrease normal and intracellular collagen synthesis of fibrotic hepatocyte, decrease extracellular collagen production in subcultured HSC and normal hepatocyte, inhabit fibroblast collagen production and intracellular collagen production in HSC to some extent, but no action on intracellular collagen synthesis in normal hepatocytes. It is suggested that one of important mechanisms of Fuzhenghuayu decoction action on liver fibrosis may be the inhibition of liver collagen production.

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 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(6):550-552

Brief Reports

Three-dimensional structure of lymphatics in rabbit stomach *

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Subject headings stomach; corrosion casts; lymphatics; three-dimensional structure

INTRODUCTION

Recently, the stomach lymphatics have been studied, but there are different opinions on the lymphatic distribution of the stomach layers[1-6]. There has been no reported in China. Discribing the three-dimensional organization of the stomach lymphatics and revealing the correlation of the three-dimensional and the two-dimensional and organization.

In our study in the rabbit with the lymphatic corrosion cast with Mercox and semithin section methods we investigated the relationship of the three-dimensional organization with the drainage of the stomach lymphatics, which may provide the evidences of lymphology, pathology and the clinical medicine.

MATERIALS AND METHOD

Twelve rabits of both sexes were used, two of them, undergone the procession of the seemitin section of electron microscopy, were observed under light microscopy. The other ten were used for the lymphatic corrosion casts.

The Mercox (CL-2B-5, Japan Velene Hospital, Tokyo,) diluted to 25%-30% (V/V) with methyl methacrylate monomer was injected in and around the mucosal submucosal, layers of the stomach. Shortly before the injection, a curing agent (MA. Japan Vilene Hospital Tokyo) was added to the injection medium to give a concentration of 1% (W/V). The injected parts of the stomach were removed and placed in a hot water bath (60℃) for 3hrs. They were put in concentrated NaOH (15%-20%) at about 60℃ until tissue elements were completely corroded away. The lymphatic corrosion casts were cut into blocks and observed under a SEM (S-520) (with an accelerating voltage of 10-15kv).

RESULTS AND DISCUSSION

The lymphatic of mucosal and submucosal layers

The samples filled with resin which were in the mucosal and the submucosal layers clearly showed the three-dimensional organization of the lymphatic capillaries and the lymphatics. There was a layer of the lymphatic capillary network in the deep layer of the tunica mucosa between the bottoms of gastric gland and the muscularis mucosa. The networks extended short tube with blind ends into gastric glands. The tubers were called intergland circular cones. which were 20μm-30μm in diameters. The cones were round, hook, V and finger in shap. In the cardia and the fundus of the stomach the cones were sparse and connected to the lymphatic capillary networks of the tunica mucosa. In the body of the stomach 2 or 3 circular cones were connected in one group. The roots of the circular cones were connected to the sinus (50μm-60μm in diameters), then drained to the lymphatic capillary networks of the tunica mucosa (Figure 1).

Donini has observed the lymphatic capillary networks of the subepithelium were in the stomach pylorus. We observed the lymphatic capillaries between the bottoms of the tunica mucasa gland and the muscularies mucosa, but did not observe the lymphatic capillaries were in the subepithelium. Our observations were smilar to Han's studies[5]. We found the lymphatic capillaries were in the tunica mucoa, but no thick lymphatics. A large number of lymphatic capillaries and lymphatics were found in the tela submucosa. The lymphatic capillaries formed a coarse network. The lympahtics (vessels) also formed a coarse plexus. The corrosion casts of the lymphatic clearly showed the three-dimensional organization of the lymphatics. The diameter of the lymphatic capillary was 10 μm - 30 μm, but the lymphatic vessel's diameter was 30 μm - 100 μm.

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The size of the meshes of the lymphatic vessels were varied and interconnected, triangular, oval and polygon in shape. The semithin sections also showed rich lymphatic capillaries and lymphyatic vessels in the stomach tela submucosa. On the surface of the lymphatic casts, we found marked constrictions characteristric of bicuspid valves (Figure 2).

The lymphatics of the tunica muscularis

A rich lymphatic capillaries and lymphaties were found in the stomach tunic muscularis. Some the lymphatic capillaries of 7μm-30μm in diameter extended the short branches with blind ends. The diameter of the lymphatic was 30 μm - 80 μm. Between three muscular layers there were lymphatic capillaries and the lymphatic vessels. The lymphatics were string of beads in shape and interconnected to plexus. There were break ends of the anastomotic channels to the superficial and the deep part from the lymphatics of the tunic muscularis. It suggested that the lymphatics of the tunica muscularis were interconnected with both the lymphatics of the tela submucosa and the lymphatics of the tunica serosa. The surface of the lymphatic casts in the tunica muscularis there were folds which run parelled to the lymphatic major axis. The imprints of endothelial nuclei were denser than other layers. On the surface, we could see the transverse imprints which were induced by the smooth muscle contraction. Between the lymphatic capillaries and lymphatic vessels of the tunica muscularis there were anstomotic channels which existed in each layer of perimysium. The lymphatics and the lymphatic capillaries of the tunica muscularis were seen in the histological sections.

$O_{ctrovekhov}$ thought that there was not any lymphatic capillary. But Nariadchikova pointed out that the lymphatic vessels and the lymphatic capillaries of the tunica muscularis only existed among the three layer's muscularis but not in each muscular layer. Our experiment proved that in the connective tissue there were both lymphatic capillaries and lymphatic vessels in each smooth muscularis layer. We also observed the lymphetic capillaries among perifascicular parts of each muscular layer.

The lymphatics of the tunica serosa

There were both the lymphatic capillaries and the lymphatic vessels in a deep part of the tunica serosa (Figure 3). The meshes of the lymphatic capillary nextwork and the lymphatic plexus in the layer were larger than those of the ttela submucosa and the tunic musecularis. The meshes presented in willow leaf, oval or trianglar shape. The lymphatic capillaries and the lymphatic vessels were also observed in the semithin sections of the layers under the light microscopy.

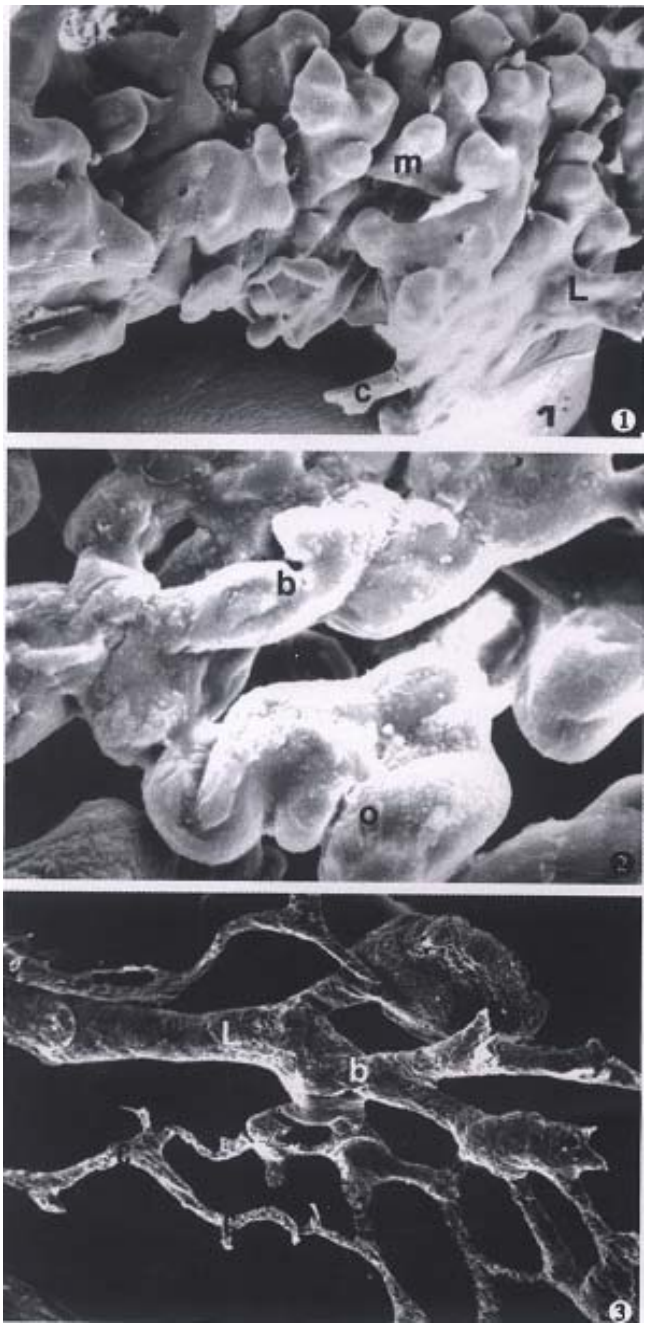
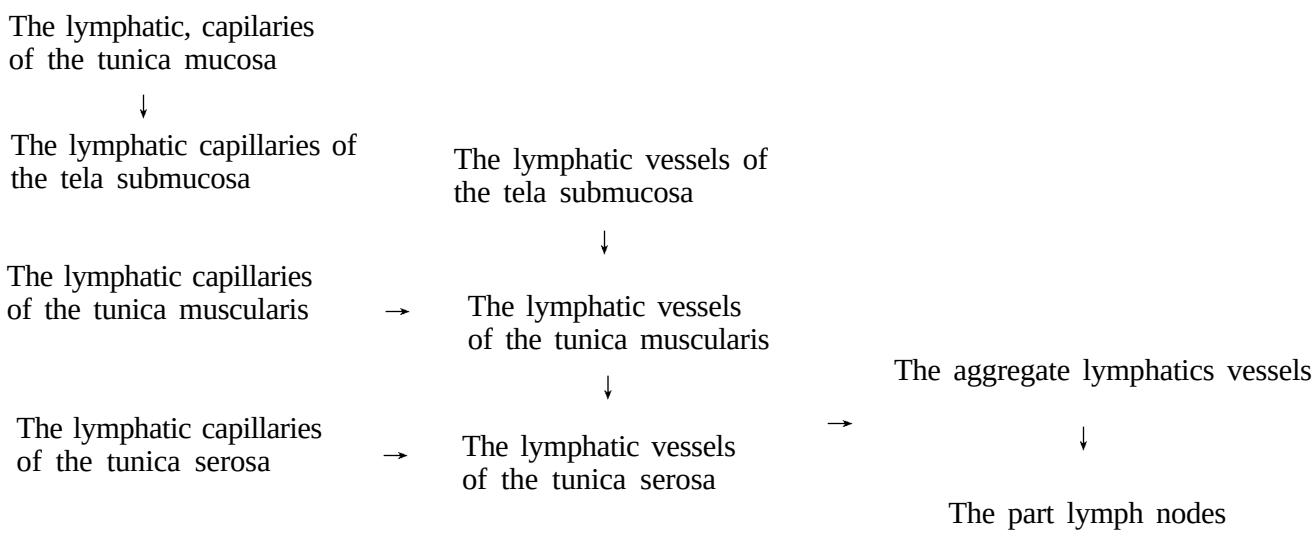


Figure 1 The intergland circular cones of tunica mucosa (m) the lymphatic capillaries (c) and the lymphatics (L) of the tela submucosa. SEM×150

Figure 2 The lymphatic networks of the tela submucosa. The constriction (b) of the cast surface presenting the bicuspid valves; the ovar or fusiform indentations (o) presenting the endothelial nuclei of the lymphatic. SEM×550

Figure 3 The lymphatic capillaries (c) and the lympatic vesseles (L) of the tunica serosa. The constriction (b) presenting the impression of the bicaspid valves. SEM×200

Donin reported that only in the curvatura veutriculi minor and major did the lymphatic



The drainage correlation of the lymphatics of all the layers.

capillaries of the tunica serosa exist. Rakhan tought the lymphatic capillaries of the tunica serosa only existed in the parts of pylorus. Ohtani[3] pointed out that only the lymphatic vessels existed in the longitudinal muscle layer. In our lymphatic casts and semithin setions we observed both the lymphatic capillaries and the lymphatic vessels existed in the tunica serosa.

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