The Editor's Note

Strengthen international academic exchange and promote development of gastroenterology

MA Lian-Sheng and PAN Bo-Rong

Approved by the China state Committee of Science and Technology in January 1998 (Document No. 027), China National Journal of New Gastroenterology has been renamed as World Journal of Gastroenterology and will be published bimonthly and distributed world wide. This is an important turning point in the publication of this Journal.

Since the Journal was established on October 1, 1995, 229 articles, including 8 editorials, 165 original articles (72.0%), 5 reviews, 6 case reports and 45 brief reports have been published in 9 issues of 3 volumes. Seventy-four papers were funded by the National Natural Science Foundation of China, accounting for 44.8%. Through its content, the Journal reflected the achievements in China' s gastroenterology made in this period and promoted the international academic exchange in the field of digestion. The Journal was distributed worldwide, covering 32 provinces and autonomous regions domestically and many developed countries as well, including UK, USA, Japan, France, Germany, etc. The Journal has been listed in 8 major indexes of China.

The policy of the World Journal of Gastroenterology is to strengthen the global academic exchange in the field of digestion of both modern medicine and traditional medicine, enhance the development of gastroenterology, and make contributions to protecting the people' s health in the world.

The principle and standard of the publication are high quality and practical. Being a peer-reviewed periodical, the Journal will publish the new achievements, in both experimental researches and clinical studies of digestion of modern medicine and traditional medicine. Priorities are especially given to the original articles of international competitive significance, editorials on the hot topics of gastroenterology, and original articles on the major projects funded by the nation. The Journal is also featured by columns of commentary, reviews and brief reports about new methods, new technics, new experience or information.

We greatly appreciate the extensive concern, and opinions and suggestions about the Journal from the gastroenterologists in the world.

On the occasion of the renaming of the Journal, we would like to extend our sincere gratitude to all our readers, members of the international advisory board and the editorial board, and leaders at different levels.

We are especially grateful to Prof. WU Jie-Ping, Vice-Chairman of the Standing Committee of the National People's Congress of the PRC and academician of Chinese Academy of Engineering and Chinese Academy of Sciences, for inscribing the Chinese name of the Journal. We also owe the smooth publication of the Journal to the great help by Prof. XU Jia-Yu, the Honorary Editor-in-Chief (Ruijin Hospital, Shanghai Second Medical University), Prof. LU Han-Ming (Xinhua Hospital, Shanghai Second Medical University), Prof. WU Xie-Ning (Shanghai First People's Hospital), Prof. MA Jing-Yun, the Associate Editor-in-Chief (senior editor of the Chinese Medical Association) and so on. Let us goforward hand in hand and strive to make this Journal an important forum for international academic exchange and contribute to the development of global gastroenterology.

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Significance of vascular endothelial growth factor messenger RNA expression in gastric cancer

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Subject headings stomach neoplasms; RNA, messenger; neoplasm metastasis; gene expression; endothelial growth factor

Abstract

AIM To study VEGF mRNA expression in gastric carcinoma and to clarify the association of its expression with the clinicopathologic features of the disease.

METHODS *In situ* hybridization (ISH) and histochemistry were used to examine and analyze the expression of VEGF mRNA and antigen, and microvessel count (MVC) in 28 cases of gastric carcinomatous tissue in combination with clinical materials.

RESULTS Ninteen of 28 gastric carcinomas were positive for VEGF mRNA. VEGF mRNA was mainly expressed in malignant cells and not in normal epithelium of gastric mucosa. Its expression was further increased in tumor cells adjacent to tumor necrosis zones, where stromal cells expressed VEGF mRNA occasionally. There was a close correlation between MVC and VEGF mRNA positivity (P <0.005). High VEGF mRNA levels were significantly associated with serosal invasion, lymph node metastasis and TNM staging (P < 0.05, respectively).

CONCLUSION VEGF mRNA expression is associated with tumor invasion and metastasis by stimulating angiogenesis in gastric carcinoma.

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INTRODUCTION

Solid tumors are composed of two distinct but interdependent compartments, the malignant cells themselves and the vascular and connective tissue stroma that they induce and in which they are dispersed. Stroma provides the vascular supply that tumors require for obtaining nutrients, gas exchange, and waste disposal. Thus, any increase in tumor mass, either primary or metastatic, must be accompanied by stroma formation[1].

The mechanism by which tumors induce stroma has caused considerable attention in recent years. Most work have focused on one aspect of stroma generation, angiogenesis, and have called attention to a variety of tumor-secreted "angiogenesis factor", particularly VEGF, that is likely to play an important role in both angiogenesis and other aspects of tumor stroma generation. VEGF is an M_r 34 000 - 42 000 KD, disulfide-linked glycoprotein synthesized by several human and animal cell types, both normal and neoplastic[2-4]. VEGF was originally recognized for its ability to increase the permeability of the microvasculature to circulating macromolecules. More recently, VPF has also been shown to be a selective endothelial cell mitogen, and therefore has been alternatively called VEGF[5-7]. By alternating splicing of mRNA, four different molecular species with 121, 165, 189 and 206 amino acids were determined.

VEGF was originally discovered as a tumor-secreted protein, and its role in tumor development were investigated[5,8]. It is considered to play an important role in tumor biology in at least two ways: as a vascular permeability factor and/or endothelial growth factor. As a potent permeability factor, VEGF promotes extravasation of plasma fibrinogen, leading to the formation of a fibrin network which serves as a substratum for cell migration during angiogenesis. While as an endothelial growth factor, VEGF stimulates endothelial cell proliferation and is likely to induce the formation of new blood vessels[9].

VEGF is synthesized and secreted by a variety of tumor cells in tissue culture and by several transplanted animal tumors *in vivo*[7,10]. Elevated expression of VEGF in human tumors have been reported in most tumors. However, there has been few reports on the VEGF expression in gastric carcinoma.

In this study, we examined the VEGF expression in gastric carcinoma by means of *in situ*

hybridization (ISH) and compared it with the pathological features of gastric carcinoma in order to determine whether VEGF is overexpressed in gastric carcinoma compared with the normal stomach tissues and its role in the invasion and metastasis of gastric carcinoma.

MATERIALS AND METHODS

Subjects and tissue samples

Twenty-eight patients with gastric carcinoma who underwent surgical resections at Ruijin Hospital were studied, including 19 men and 9 women, aged from 38 to 78 (mean age 56.2 ± 11.9) years. None had received chemotherapy or radiotherapy before surgical resection. A tumor sample and a normal part of the stomach were obtained during surgical resection. The samples were each divided into two pieces. One was subjected to fixation in 10% formalin for histological examination and immunohistological test, and the other was used for ISH, i.e., tissues were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4℃ and were then transferred to 30% sucrose in PBS, pH 7.4, overnight at 4℃. The tissues were then frozen in OCT (Miles Inc.) and stored at - 70℃ until use for VPF/VEGF mRNA expression analysis. Pathological features of the patients were obtained from the pathological reports and clinical records. Histological analysis of the tumor was done by experienced pathologists without knowing the analytical results. The differentiation grading was based on the predominant findings.

Preparation of single-stranded RNA probe for ISH

The pBluescript II SK + construct with the VEGF insert (a gifted from Dr. Yamauich, Japan) was linearized with B*am* H I and transcribed *in vitro* from the T_7 polymerase promoter to yield the antisense RNA probe. The same construct was linearized with E*co* R I and transcribed from the T_3 polymerase promoter to yield the sense (control) probe. The transcription reaction was performed in the presence of 0.35mM digoxigenin-UTP (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) to yield digoxigenin-labeled RNA probe.

In situ hybridization

In situ hybridization was performed using a digoxigenin nucleic acid detection kit with some modifications. Frozen sections were cut at 10 μm, fixed for 20min in 4% paraformaldehyde/0.1M PBS, and rinsed in PBS. Specimens were then treated with 0.1% proteinase K, fixed in 4% paraformaldehyde/0.1M PBS again, acetylated in 0.1M triethanolamine and 0.25% acetic anhydride, and dehydrated through a graded ethanol series. Sections were prehybridized for 2 hours at 53℃ with 50 μl of prehybridization fluid containing 50% formamide, 0.3M NaCl, 10mM Tris-HCl(pH 8.0), 1mM EDTA, 10% dextran sulfate, 1 × Denhardts solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone), and 0.025% salmon sperm DNA in a humidified chamber. After the prehybridization, hybridization was performed in buffer containing prehybridization fluid and 1.0 mg $\cdot L^{-1}$ of either antisense or sense probe at 53℃ for 16 hours. Next, sections were washed at 37℃ for 30min in 4×SSC (150mM NaCl and 15mM sodium citrate) and 30min in 2 × SSC/0.05%SDS, digested with RNase (20 mg $\cdot L^{-1}$) at 37℃ for 15min, and washed again at 37℃ for 30min in 2 × SSC/0.05% SDS, and washed at 37℃ for 30min in 1 × SSC, 0.5 ×SSC, respectively. Then, blocking was performed in blocking buffer containing 1% BSA for 30min. After the blocking buffer was removed, the solution containing 1 : 1000 sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase and 1% BSA was placed on each section. Sections were placed in a humidified chamber and incubated at room temperature for 1 hour. After the sections were rinsed, a coloring reaction was performed using 225 μg$\cdot mL^{-1}$ nitroblue tetrazolium salt and 175 mg $\cdot L^{-1}$ 5-bromo-4-chloro-3-indolyl-phosphate. When the color reaction was appropriate, stop it by washing in buffer solution. The section was washed, dehydrated and mounted.

Microvessel count

The methods of microvessel staining and counting were as described previously[11].

Statistical analysis

The clinicopathological features between VEGF expression and nonexpression groups were analyzed by Fisher's test. The correlation between MVC and VEGF expression was evaluated with Student' s *t* test.

RESULTS

Expression of VEGF mRNA in gastric cancer

Twenty-eight cases of human gastric carcinoma were studied by ISH with both antisense and sense (control) probes to VEGF. VEGF mRNA was expressed in the malignant epithelium of 19 of the 28 patients (19/29, 67.9%). Generally, VEGF mRNA was distributed homogene ously and intensely throughout the tumor. VEGF was expressed by tumor cells under microscopy (Figure 1). However, it is noteworthy that in the periphery of necrotic foci of the gastric cancer, labeling with the VEGF antisense probe was distinctly intensified in tumor epithelium adjacent to the necrotic foci. The tumor stroma cells also labeled for VEGF mRNA. Stromal labeling was distinctly focal as compared to the labeling regularly observed over tumor cells and usually occur red in stroma immediately adjacent to

the foci of overt tissue necrosis. Labeled stromal cells included fibroblasts and smooth muscle cells. However, vascular endothelium did not express detectable VEGF mRNA. No specific cellular labeling was seen with the VEGF antisense in normal epithelium of gastric mucosa from these cases, nor did tumors label with the VEGF sense probe.

Comparison of clinicopathological findings and VEGF mRNA expression

VEGF mRNA expression in gastric carcinoma was compared with the clinical and histological features of the tumors. The comparison between VEGF mRNA expression groups and nonexpression groups is shown in Table 1.

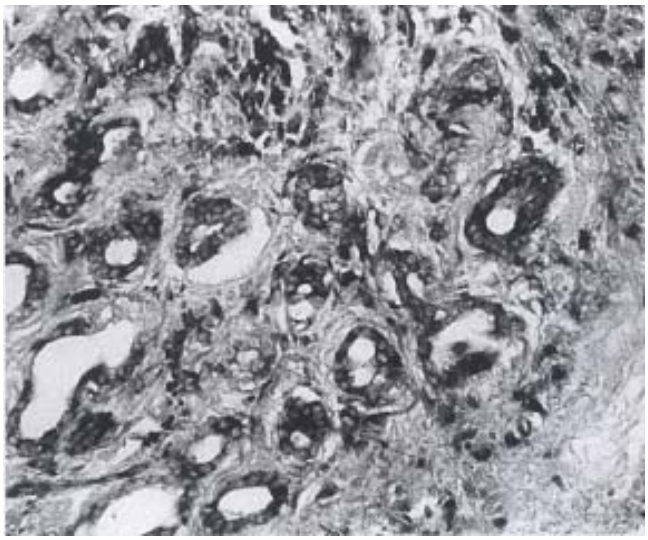

Figure 1 ISH with antisense probe to VEGF of a gastric adenocarcinoma (×200).

Table 1 Comparison of clinicopathological features of gastric carcinoma between VEGF mRNA expression groups and nonexpression groups

Characteristics	VEGF mRNA(+)	VEGF mRNA(-)	*P*
Vascularity			
MVC	18.1±7.8	12±5.9	<0.005
Serosal invasion			
Positive	16	3	<0.005
Negative	3	6	
Lymph node metastasis			
Positive	17	4	<0.05
Negative	2	5	
Histologic type			
Differentiated	2	2	<0.05
Undifferentiated	17	7	
Other metastasis			
Positive	3	0	<0.05
Negative	16	9	
TNM staging			
I	1	4	
II	9	3	<0.05
III	6	2	
IV	3	0	

VEGF mRNA expression was significantly correlated with serosal invasion and lymph node metastasis, although no correlation was found between its expression and histologic type, and other metastasis. Incidences of VEGF mRNA expression were increased as the pathologic stage progressed.

Correlation between VEGF mRNA expression and MVC

Factor VIII was stained in tumor vascular endothelial cell. Microvessel counts varied from 3 to 38 counts/ ×200 field (average, 15.6 counts). Table 1 shows the correlation between the MVC and VEGF mRNA status. The MVC in VEGF mRNA positive tumors was significantly higher than that in VEGF nonexpression tumors.

Relationship between VEGF antigen and its mRNA expression

VEGF antigen was mainly identified in the cytoplasm of cancer cells. Expression rate of VEGF antigen was 46.4% (13/28). Although it was lower than that of VEGF mRNA, there was a significant correlation between their staining intensity (r = 0.487, $P < 0.05$).

DISCUSSION

Brown *et al*[12] examined 2 cases of gastric adenocarcinomas on VEGF mRNA and observed a detectable signal for VEGF in all them using ISH. In this paper, 19 of the 28 primary human gastric carcinoma expressed VEGF mRNA as judged by ISH, the expression rate was 67.9%. VEGF mRNA was mainly detected in malignant glands but not in normal epithelium. This is different from the phenomena that VEGF mRNA expression was detected both in tumor and normal tissue in liver[13] and lung[14], and it is suggested that the VEGF expression seems to be a characteristic of the malignant phenotype in gastric carcinoma.

It is worth notice that there are some differences in the strongly stained areas between VEGF mRNA and antigen. Tumor cells stained strongly for VEGF antigen were observed more often in the invasion front than in the tumor center, inversely, VEGF mRNA was expressed more often in the tumor center adjacent to the zones of tumor necrosis than in the invasion front. VEGF mRNA expression elevation around the necrotic area of the tumor has been reported in several other human cancers. Rapid cell proliferation in the center of a tumor can lead to increased interstitial pressure, which may lead to compression closure of capillaries and consecutive tumor necrosis. Tumor hypoxia has been reported to increase expression of VEGF mRNA in a variety of cultured tumor cells[15]. Therefore, it is likely that, in the development of necrosis, hypoxia may increase the VEGF mRNA

expression.

Some studies had demonstrated the correlation between the expression of VEGF mRNA and tumor invasion and metastasis. Recently, Zhang *et al*[16] reported that the transfection of this growth factor gene into a tumor cell line expressing a low level of VEGF proteins altered tumor cells more angiogenesis and more progressive in the nude mouse model without change of cell growth rate *in vitro* . In this study, we observed a significant difference of MVC between VEGF mRNA expression group and nonexpression group. The incidences of serosal invasion and lymph node metastasis in tumor which expressed VEGF mRNA were higher than that of tumors with non-VEGF mRNA expression. The tumors with liver or ovarian metastasis are VEGF mRNA positive. Moreover, the rate of VEGF mRNA expression in the advanced tumors is higher than that in early stage tumors. All these results indicate that VEGF mRNA may play an important role in tumor angiogenesis, invasion and metastasis of gastric carcinoma.

VEGF mRNA expression was observed in 19 cases, but still was not detected in 9 cases. This suggests that angiogenesis is not simply controlled by the presence of VEGF but is mediated by several angiogenic inducers. To date, many angiogenic factors have been found, including basic fibroblast growth factor, transforming growth factor-β, and tumor necrosis factor α. In fact, there has been a lot of reports on bFGF expression in gastric carcinoma. Therefore, it is likely that vascularization in some tumors is related to other angiogenic factor. If VEGF is indeed responsible for gastric cancer angiogenesis, therapeutic strategies to inhibit its activity using either specific antibodies or antisense RNA may allow clinicians to treat not only the malignant cells within a tumor but also the vascular supply of the tumor. In conclusion, the identification of VEGF that correlates with angiogenesis in gastric cancer may provide a basis for targeting this angiogenic factor to inhibit vascularization of tumors.

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CCR5 gene expression in fulminant hepatitis and DTH in mice

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Subject headings CCR5 gene; DNA, complementary; gene expression; cloning, molecular; hepatitis; hypersensitivity, delayed; disease models, animal; polymerase chain reaction; immunohistochemistry

Abstract

AIMS To isolate mouse CCR5 cDNA (muCCR5) and study its expression *in vivo*.

METHODS Marathon PCR was used to isolate muCCR5 cDNA and two animal models were designed to investigate the gene expression *in vivo*, one was mouse fulminant hepatitis induced by Propionibacterium acnes (P.acnes) and the other was that with delayed type hypersensitivity reaction (DTH). A specific GST-NH2-terminus of muCCR5 fusion protein antibody F(ab')2 was prepared and clarified. RT-PCR and immunohistochemical analysis were used to observe the expression level of CCR5 gene in mice.

RESULTS A positive reaction of mouse macrophage was found in DTH but not expressed in P.acnes induced fulminant hepatitis by RT-PCR and immunohistochemical analysis.

CONCLUSION This muCCR5 expression may be involved in an allergic processmediated by cellular immunity but not acute inflammatory reaction induced by P.acnes.

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INTRODUCTION

Leukocytotaxis plays an important role in immune system surveillance and chronic inflammation. Locally produced chemotactic factors are thought to be critical in this directed migration. The chemotactic family can be divided into two subfamilies: ①the 1st subfamily having the first two of the conserved cystein residues separated by another amino acid, the CXC (α) chemokines, and the second with CC unseparated the CC (β) chemokine. The β-chemokine receptors are some G-protein coupled receptors with seven transmembrane domains and share a high degree of amino acid homology in their putative transmembrane domains. Recent researches indicated that the entry of HIV into target cells required the participation of at least two cell surface molecules: one was CD4+ which was utilized by all HIV strains as the primary virus receptor through a high affinity interaction with the viral envelope protein. However, the CD4+ alone was not sufficient for virus entry, and some additional cell surface molecules, termed cofactor, for example, CCR5, CCR3, CCR2b and fusin[1-5] were found to mediate the entry of HIV-1 into the host cells. CCR5 can express in monocytes, macrophages, and primitive T cells, and bind to β-chemokine RANTES, MIP-1α and MIP-1β. Expression of CCR5 in conjunction with CD4+ in a variety of cell types renders them permissive for infection through M-tropic envelope proteins. Meanwhile, CCR5 and CD4+ are expressed in several cells to mediate the M-tropic HIV strain envelope to form syncytia[6]. The M-tropic HIV-1 strain is most sensitive to changes in the first extracellular loop, and therefore, to understand the CCR5 expression is very important. Unfortunately, we found that under the normal condition, mouse CCR5 gene expressed only in a few cell lines and at a very low level *in vivo*. In this study, we used the model of DTH (delayed type hypersensitivity) and a fulminant hepatitis induced by P. acnes for observing the CCR5 expression *in vivo* and analyzed the mechanism of the gene expression.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney cell line 293 was cultured in DMEM (Nissui Pharmaceutical Co, LTD,) medium containing 10% fetal calf serum and 50 U/ml penicillin G and 50 μg/ml streptomycin.

Molecular cloning of mouse CCR5 cDNA

Mouse peritoneal macrophages isolated 3 days after pentose injection, and their total RNA was prepared using RNA 201 B (Cinna/Biotec, Houston, TX). Macrophage total RNA was reverse-transcribed by RT-PCR using a random primer (Takara Shuzo Co. LTD) in the presence of RNase inhibitor (Promega). Corresponding to the sequences of the highly conserved region between the second and fifth transmembrane domains within human MCP-1 and mouse MIP-1α receptors, the sense primer was (PTM_2) 5'-GCGAATTCTGGCCAT (CT) TCTGA (CT)CTGCT(CT)TT(CT)CT-3', and the antisense primer was (PTM_5) 5' GCAAGCTT (GC) A (CT) (GT) GG (AG)TTGA (CT) (AG) CAGCAGTG (AC) GT-3'. 5'-RACE and 3'-RACE reactions were performed to isolate the full-length mouse CCR5 cDNA by means of Marathon cDNA amplification kit (Clontech, CA). In brief, the first PCR reaction was carried out using primer "R1" and the primer adapter 1. The second PCR reaction was performed with the internal primer "R2" of CCR5 and the other primer adaptor 2. The specific primers of mouse CCR5 were as follows: (R1) 5'-GGATCAGGCTCAAGATGACC-3', (F1) 5'-ACACTCAGTATCATTTCTGG-3'. PCR products were digested with appropriate restriction enzymes and subcloned into pBluescript SKII $^+$ (Stratagene). DNA sequencing reaction was performed by a PCR procedure employing fluorescent dideoxynucleotides and analyzed by a model 373A automated sequencer (Applied Biosystem).

Construction of expression vector for mouse CCR5 and preparation of stable transfectant

For construction of the expression vector of mouse CCR5, the coding region of mouse CCR5 gene was amplified by PCR with specific primers and cloned into pcDNA3 (Invitrogen Corporation). The 5' primer for PCR was designed to generate Kozak sequence, and the constructs were introduced into a human embryonic kidney cell line 293 by the calcium phosphate coprecipitation method modified by Chen[7]. Transfected cells were selected in the presence of a neomycin analogue, G-418 (Life Technologies, Inc), at a concentration of 500 ng/L in complete medium.

Preparation of GST proteins fused with extracellular domains of mouse CCR5

For preparing a recombinant GST protein fused with NH_2-terminal portion of muCCR5 cDNA, the NH_2-terminal extracellular binding domain encoding Metl-Leu38 from the ORF region of muCCR5 cDNA was obtained by polymerase chain reaction, and then cloned into EcoRI and BamHI restriction sites of the GST-fusion protein expression vector pGEX-IN. The recombinant DNA was transferred into *E. coli* HB 101 competent cells (Toyobo competent) . The expression and purification of a GST fusion protein were induced with 0.1 M IPTG (isopropylthio- β-galactoside, Wako Pure Chemical Industries, LtD) for 5 hours, put on glutathione-sepharose 4B affinity column (Pharmacia Biotech AB Upsala, Sweden), and then eluted with 5mM of reduced glutathione.

Preparation of polyclonal antibodies F(ab')$_2$ of GST protein fused with NH2-terminal portion of muCCR5

Two New Zealand white rabbits were immunized with 100 μg of the GST-NH_2-terminal of muCCR5 fusion protein in CFA (Iatron, Tokyo, Japan); first time, the footpads were injected, and other 9 times at biweekly intervals were given s.c.. One week after the final immunization, rabbits were bled, sera were obtained, and fractionated into IgG by a protein A agarose column (Pharmacia-Biotech, Upsala, Sweden). A portion of the IgG fraction was further digested with pepsin (Sigma Chemical, St. Louis, MO) and the F (ab')$_2$ fragment was obtained by sequential chromatographies using a protein A affinity and a sepharose 12gel filtration column as previously described[8].

DTH reaction and RT-PCR amplification of muCCR5

BALB/c female mice of 6 - 7 weeks were used. The hair of abdomen were shaved, 5% Picryl Chloride 100 μl was painted on the surface of abdomen, after 5 days 1% picryl chloride was painted on the right ear, 24 and 48 hours later the effect of DTH was evaluated, and then the tissues were taken for preparing total RNA with RNA 201. RT-PCR was run according to Normua[9] (94℃ for 1min, 55℃ for 1min, and 72℃ for 1.5min, with a total of 30 cycles).

Induction of fulminant hepatitis in mice

Mice were injected with 1mg of heat-killed P.acnes into the tail vein. Seven days later the indicated dosages of LPS were administered intravenously[10]. At 3 hours from LPS challenge, three to four mice were killed to obtain the liver. Liver tissues were fixed in 4% paraformaldehyde for 2 hours before being transferred to 70% ethanol and subsequent paraffin embedding.

Immunohistochemical analysis of liver and spleen tissues

For immunohistochemical analysis, paraffin-embedded tissues were dewaxed with histo-clear (National Diagnostics, Tokyo, Japan) and dehydrated through graded concentrations of ethanol. After being treated with trypsin and blocked with 1% skim milk, the tissue sections were covered with 40ng/L of anit-muCCR5 antibody F

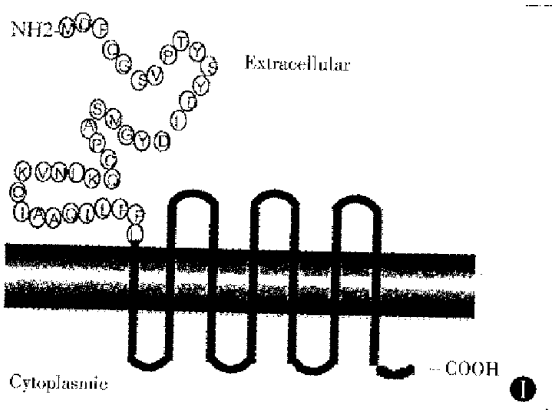

Figure 1 The first extracellular loop amino acid sequence and transmembrane structure of muCCR5. The amino acid fragment from M to L is located in the first loop of extracellular and it is the binding domain of mouse CCR5 for ligand (MIP-1α, MIP-1β and RANTES).

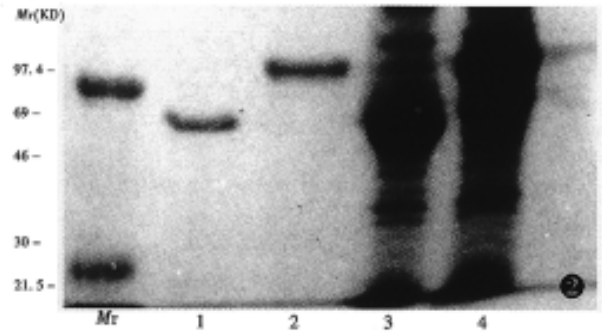

Figure 2 Analysis of recombinant GST-muCCR5-NH2 terminal fusion protein.
Mr: MW marker, 1, 2: products purified by glutathione sepharose, 3, 4: products not purified, 1, 3: expression products induced by IPTG

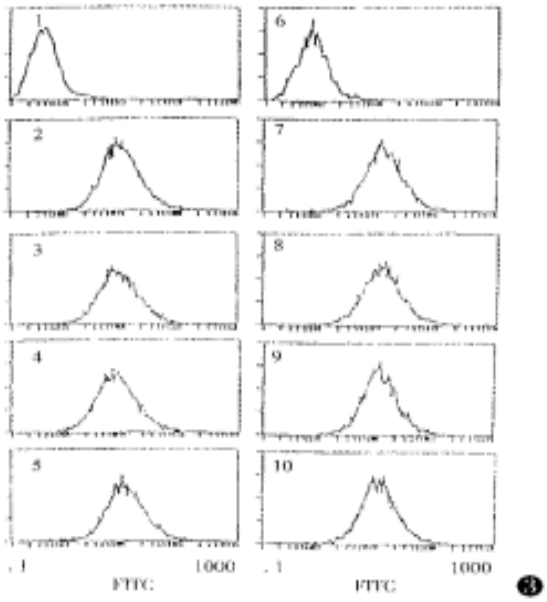

Figure 3 Flow cytometric analysis of specificity of anti-muCCR5 antibody F(ab')2.
F(ab')2 volume is 40mg / L, blocking reagent volume is 100mg / L.
1. 293 cells; 2. transfected cells of muCCR5. Transfected cells were blocked by:
3. GST; 4. muIL-8; 5. huFusin; 6. GST-muCCR5-NH2; 7. muCCR1; 8. huCCR2; 9. huCCR3 and 10. huCCR4.

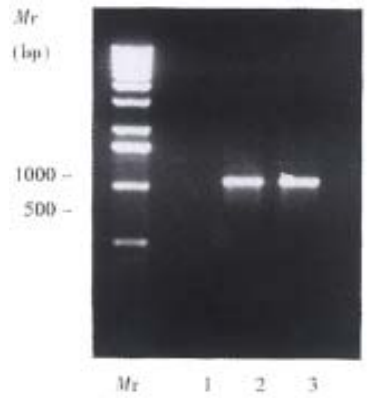

Figure 4 RT-PCR analysis of DTH reactions.
muCCR5 specific primers were used to amplify the cDNA from spleen cells. Lane.
1. BALB / C moase fulminant hepafifis induced by P. acnes, Lane 2.24 hs result after 1% picryl chloride stimulation. Lane 3.48 hs result after 1% picryl chloride stimulation.

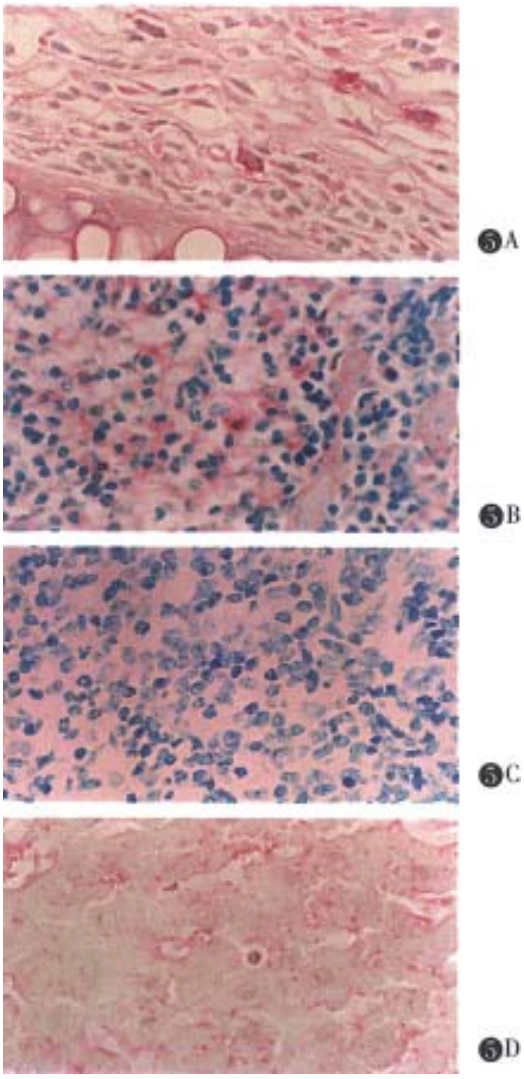

Figure 5 Immunohistochemical analysis of DTH reaction.
A. Ear: Most of the macrophages in subepidermal interstitial tissue and in the local capillary were obviously positive in their cytoplasm for the staining.
B. Spleen: There were plenty of reticular-macrophage in red medulla. A few slightly positive cells, however, could be seen scattered there.
C. The negative result was used by anti-GST F(ab')2.
D. Liver: The cytoplasm of Kupffer cells were slightly stained by the dyes.

$(ab')_2$ and GST over night at 4℃, incubated with biotinylated swine anti-rabbit IgG, and then reacted with ALP substrate solution (Vector Laboratories, Burllingame, CA) containing 1mM levamisole and solution I, II, III for 40min at room temperature. Finally, the sections were rinsed and counterstained with 1% methyl green.

RESULTS

Molecular cloning of muCCR5 cDNA and preparation of a fusion protein of NH_2-terminus of muCCR5 with the C-terminus of GST

A full length cDNA sequence and amino acid sequence of muCCR5 was cloned by Marathon PCR from the poly $(A)^+$RNA of mouse peritoneal macrophages. The ORF region of muCCR5 consisted of 355 amino acid, and had a 82% homology with human CCR5. A GST fusion protein of the NH_2-terminal 38 amino acids of the first loop of extracellular part of muCCR5 was expressed as cytoplasmic protein in *E. coli* (Figure 1). A corresponding fusion protein of 30kD and GST molecule of 26kD were observed on 15% SDS-PAGE with Coomassie blue-staining (Figure 2). In general, 3mg-5mg GST-NH_2-terminus of muCCR5 fusion protein was produced from 200 ml *E. coli* solution.

Determination of the specificity of anti-GST-NH_2-muCCR5 antibody

An experiment was performed to examine the cross reaction of the anti-muCCR5 $F(ab')_2$ with other receptors by means of the muCCR5 293 transfectant, and the results showed that the anti-muCCR5 F ($ab')_2$ could bind with GST-NH_2-muCCR5 fusion protein but not human CCR1, CCR2, CCR3, CCR4, Fusin and mouse IL-8 (Figure 3), suggesting the specificity of the anti-muCCR5 F $(ab')_2$ to muCCR5.

RT-PCR analysis

The expression of muCCR5 mRNA in DTH was confirmed by RT-PCR, with the sense primer 5'-ATGGATTTTCAAGGGTCAGTTC-3' and antisense primer 5'-TCATAAACCAGTAGAAACTTC-3'. The results showed that after induction with 1% picryl chloride for 24 and 48 hours, muCCR5 could express in spleen cells and the negative result was found in fulminant hepatitis induced by P.acnes (Figure 4).

Immunohistochemical analysis

In the normal mice, CCR5 expression level was very low or nil, and muCCR5 was not expressed in fulminant hepatitis induced by P.acnes reaction (unpublished data). The Immunohistochemical analysis revealed that muCCR5 clearly expressed on ear at the point of picryl chloride induction (Figure 5A). A weakly positive result was found in spleen macrophages in DTH reaction induced by picryl chloride (Figure 5B), but a negative result of DTH reaction induced by picryl chloride was found in normal experiment (Figure 5C) and a liver Kupffer cells also showed a positive result in DTH reaction (Figure 5D).

DISCUSSION

In this study we first cloned mouse CCR5 full length cDNA sequence from peritoneal macrophages ploy (A) + RNA with Marathon PCR, then constructed the fusion protein of GST with 5' terminal extracellular binding domain 38 amino acid of muCCR5 and successfully have it expressed in *E. coli*. In the Northern analysis, we could not find expression of muCCR5 in vivo. In order to understand the expression of CDR5 in vivo, two animal models were designed, one was mouse fulminant hepatitis induced by P.acnes and the other was that with delayed type hypersensitivity reaction (DTH). In previous studies, P.acnes induced transient increase in serum TNF-a levels but not those of IL-1ra, IL-1 and IL-6. However subsequent LPS challenge induced the elevated serum levels of all these cytokines and the peak serum IL-1ra level was 20 times that of serum IL-1 levels. Immunohistochemical analysis demonstrated that IL-1ra was predominantly produced by hepatocytes during the priming phase by P.acnes and eliciting phase by LPS challenge[10]. The responsiveness of alveolar lymphocytes to recombinant IL-2 was evaluated by ^{3}H-thymidine uptake in the presence and absence of P.acnes. P. acnes stimulates IL-2 production and IL-2 receptor induction in alveolar lymphocytes from patients with active sarcoidosis[11]. In our study of the expression of muCCR5 in P.acnes induction, we did not observe a positive reaction, which indicated that muCCR5 gene expression did not involve an acute inflammatory process. In the DTH reaction, the results showed that muCCR5 had expressed in mouse macrophages. No matter some factors might induce a DTH condition, in this case, cell immunity mediated CCR5 gene expression and HIV-1 in infected macrophages more easily. This is a interesting problem which needs further studies.

There have been some reports about the DTH reaction, for example, RANTES produced by IFN-α and TNF-α induction has already been described in a number of studies and clearly IFN-α and TNF-α have a synergic action. INF-d stimulated macrophage to express RANTES and TNF-α stimulated cells to express RANTES gene. It is well known that RANTES is a ligand of CCR5. In the DTH state, macrophages not only express ligand but also express its receptor. In this case, if the ligand binds to its receptor, what physiological changes will happen? Or the ligand (RANTES) thus produced may induce receptor expression; this is another interesting

problem. Th-2 cytokines such as IL-4 and IL-10, have been demonstrated to inhibit DTH granule formation and increased production of these cytokines was associated with impaired DTH reaction[11-15]. Th-2 cytokines may affect CCR5 expression of macrophages, because a high concentration of Th-2 cytokines can alter the recruitment of immune cells to DTH granules at least partly by inhibiting CCR5 expression.

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Relationship between tumor necrosis factor-α and liver fibrosis

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Subject headings tumor necrosis factor/ metabolism; laminin/blood; hyaluronic acid/blood; liver cirrhosis/blood

INTRODUCTION

To intrestigate the relationship between tumor necrosis factor-α and liver fibrosis in patients with chronic liver disease.

METHODS

Radioimmunoassay was made in 20 patients with mild chronic hepatitis (CMH), 20 patients with severe chronic hepatitis (CSH), 51 patients with liver cirrhosis (LC) and 32 normal persons to determine the contents of tumor necrosis factor-α (TNF-α), laminin (LN) and hyaluronate (HA) in serum. The changes in and relationship between TNF-α, LN and HA were analyzed. The TNF-α and collagen III were determined using immunohistochemical studies in liver tissues from 32 persons including 7 normal persons, 3 patients with MCH, 5 patients with SCH and 17 with LC.

RESULTS

TNF-α, LN and HA levels in serum of CSH and LC patients were significantly higher than those in healthy controls (SCH: 1.11 ± 0.59,130.7 ± 17.2,219.1 ± 121.3; LC:0.92 ± 0.66,156.8 ± 31.7, 400.5 ± 183.7, P<0.05 - 0.01), which increased gradually, and correlated positively with each other in all patients with liver diseases (n = 91, γ = 0.3149,P < 0.01). TNF-α contents showed a remarkably positive correlation with HA and LN levels in CMH and CSH (LN: n = 40, γ = 0.3404,P < 0.05; HA: n = 40, γ = 0.3847, P < 0.05). The total collagen content of MCH, SCH and LC increased gradually in liver biopsy specimens. The number of TNF-α positive cells increased significantly in liver tissues from patients with SCH and LC (62% ;45% ; P < 0.01). TNF-α positive cells were mainly located in the periportal areas.

CONCLUSION

TNF-α may be related to liver fibrosis, and might promote liver fibrosis.

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CONCLUSION

TNF-α may be related to liver fibrosis, and might promote liver fibrosis.

Induction of apoptosis of lymphocytes in rat mucosal immune system *

CHEN Xue-Qing, ZHANG Wan-Dai, SONG Yu-Gang, ZHOU Dian-Yuan

Subject headings mucosal immune system; cycloheximide; proliferating cell nuclear antigen; immunohistochemistry

Abstract

AIM To undergo apoptosis during negative and positive selection processes in rat mucosal immune system which are implicated in the pathogenesis of various mucosal diseases.

METHODS Female Sprague-Dawley rats were given protein synthesis inhibitor, cycloheximide, intravenously or intraperitoneally, an apoptosis was recognized by morphological hallmark under light and electronmicroscopy, and the expression of proliferating cell nuclear antigen was visualized immunohistochemically.

RESULTS The apoptosis of mucosal lymphocytes in the digestive tract, as well as in trachea, uterus and lacrimal gland was induced by cycloheximide (>1.0 $mg \cdot kg^{-1}$ body weight), which were located mainly in lamina propria and germinal centers of lymphoid nodules. At the same time, a portion of crypt epithelial cells of proliferating zone in small and large intestine, and the epithelial cells in genital tract were also found to undergo apoptosis. Immunostainings showed that apoptotic cells expressed proliferating cell nuclear antigen.

CONCLUSION Apoptosis of lymphocytes in mucosal immune system can be induced by cycloheximide. This model will facilitate the understanding of normal mucosal immune system and its role in the pathogenesis of related diseases such as inflammatory bowel diseases.

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INTRODUCTION

Mucosal immune system represents the most important humoral immune system of the body quantitatively[1,2]. In adults, about 80% of immunoglobulin producing immunocytes are located in the intestinal lamina propria showing the dominant role of the gut as an activated B-cell organ[1-3]. Antigenic priming of organized gut-associated lymphoid tissues, mainly includes the Peyer's patches, and may give rise to specific secretory immunity in not only the gut, but also the respiratory tract, lacrimal, salivary and lactating mammary glands[2]. The main function of the mucosal immune system is to protect the host against pathogenic bacteria and viruses to which the mucosal surfaces are exposed. The mucosal immunocytes play an important role in the pathogenesis of inflammatory bowel diseases(IBD), gluten enteropathy and Sjögren' s syndrome, although the mechanisms have not been elucidated yet[4,5].

Apoptosis (programmed cell death) is a process genetically controlled leading to cell suicide[6]. Peripheral T and B lymphocytes, as well as their precursors in the central lymphoid organs, may undergo apoptosis during the negative and positive selection processes. Abnormality of apoptotic machinery within the cell results in the development of various types of diseases. Resistance to apoptosis may be coupled to autoimmune diseases, and leukemia. In contrast, elevated apoptotic decay of lymphocyte has been shown to be involved in lymphopenia caused by human immunodeficiency virus infection[7]. Therefore, it is reasonable to expect that induction of apoptosis in immune system will facilitate better understanding of immune system and its role in related diseases.

In this experiment, we used a protein synthesis inhibitor cycloheximide (CHX) to establish a method for rapid induction of apoptosis of immuocytes in rat mucosal immune system. We focused our research on digestive tract as well as on trachea, uterus and lacrimal glands.

MATERIALS AND METHODS

Reagent

Cycloheximide was purchased from Serva. The monoclonal antibody against proliferating cell nuclear antigen (PCNA) used for immunostaining were from ZYMED Lab, Inc. VECTASTAIN® ABC kits were products of VECTOR. 3'-

diaminobenzidine hydrochloride (DAB) and pepsin were purchased from Sigma. All other chemicals were of regent grade.

Animal model

Female Sprague-Dawley rats (PLA Animal Center of South China, Guangzhou) weighing 200 ± 20 g were maintained on a standard laboratory diet and given water ad libitum. Twenty-four hours before experiment, they were deprived of food, but accessible to water freely. After anaesthetized with ether, the rats were injected with CHX (dissolved in 0.9% sodium chloride) intravenously or intraperitoneally at dosage of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10 mg · kg^{-1} body weight. Controls were given an equal amount of saline. Rats were sacrificed at various time points after treatment (1,2,3 and 4 hours).

Histologic examination

The tissues collected from lacrimal gland, uterus, trachea, and digestive tract (including stomach, duodenum, jejunum and ileum and colon, small portions (0.5 cm × 0.5 cm) were fixed immediately in 4% buffered-paraformaldehyde (pH 7.2) and embedded in paraffin. These were cut at 4 μm thickness and stained with hematoxylin and eosin (H & E). Apoptotic cells were identified morphologically[6,8] for shrinkage of the cells, chromatin condensation, formation of apoptotic bodies and absence of inflammation. Apoptosis incidence could be calculated randomly by counting the apoptotic bodies in lamina propria of uterus[8]. At least 200 cells per sec were counted, and three to four rats per group were used.

Electronmicroscopic examination

After the rats were sacrificed, the specimens were immediately fixed in 2.5% glutaraldehyde buffered in 0.1 M- PBS (pH 7.2) at 4℃ for at least 2h. Tissues were washed with the same buffer and postfixed in 1% osmium tetroxide in phosphate buffer at 4℃ for 2h, then dehydrated in graded acetone and embedded in Epon resin. After stained with uranyl acetate and lead citrate, the ultrathin sections were examined under the electronmicroscope (JEOL, JEM, 1200-EX, Japan).

Immunohistochemistry

Paraffin sections were deparaffined, rehydrated and digested with 0.001% pepsin in 0.01N hydrochloric acid at 37℃ for 10min, and rinsed in 0.01 M PBS (pH 7.2) for another 10min. The tissues were then incubated in chronological order with a primary anti-PCNA antibody (diluted 1 : 100) at 4℃ overnight, a biotinylated-secondary antibody at 37℃ for 30min, and avidin-biotin complex for development at 37℃ for 45min. During incubation, the sections were washed for 10min in PBS. Staining was developed in DAB-hydrogen dioxide and sections were counterstained with hematoxylin. Primary antibody was omitted in negative controls.

RESULTS

Apoptotic cells were rarely found in normal mucosal of digestive and respiratory tracts, lacrimal gland and reproductive system, even in the germinal centers of lymphoid nodules. After the rats were given CHX, apoptosis was found in the mucosa of these organs with characteristic features, and the apoptotic tissues were devoid of inflammatory response at any time point (Figure 1 A, B, C, D, Figure 2 A, B, Figure 3 B). The induced lymphocyte apoptosis was scattered in lamina propria and clustered in lymphoid nodules. At the same time, the apoptosis of intraepithelial lymphocytes (IEL), particularly in digestive tract, was also observed. Besides, a portion of the mucosal epithelial cells were found dead, including crypt epithelial cells in the small and large intestines (Figure 2 A) and epithelial cells of uterus. However, the epithelial cells lacrimal glands and trachea were still normal (Figure 1 B, C). The apoptosis of epithelium in the digestive tract was within proliferating zone of crypt epithelium (Figure 2 A, B). Despite these changes, the epithelium remained in integrity, and no erosion or ulceration were seen macroscopically. Microscopically, vascular changes were found in lamina propria with capillary dilatation and the vessels packed with erythrocytes, and no thromb was observed.

Apoptosis was defined as condensation and margination of chromatin, contraction of cytoplasm, and cell shrinkage, and the mitochondria could be seen[6,8]. In this study, at the early apoptotic stage, the mitochondria was well preserved, and its number increased as well (Figure 3B). Activated macrophages phagocytosed apoptotic bodies (Figure 1B, C). Some apoptotic bodies of lymphocytes and epithelial cells were phagocytosed by the neighboring epithelial cells.

PCNA is an important index proliferating cells[9]. The immunostaining showed strong positive expression of PCNA in lymphocytes of germinal centers, crypt epithelial cells of proliferating zone of small and large intestines (Figure 2A,B), and epithelial cells of reproductive system. The scattered lymphocyte in lamina propria in lymphoid nodules, especially in the gut were also positively expressed (Figure 3B). Fragmented nuclei were still found expressed with PCNA. Therefore, the PCNA expression was in consistent with the induced apoptosis. However, some of the lymphocytes and epithelial cells with positive PCNA expression appeared normal morphologically (Figure 3A, B). On the other hand, the cells with negative PCNA expression failed in apoptosis (Figure 3A, B), even with large dosage of CHX(>10.0 mg·kg^{-1}).

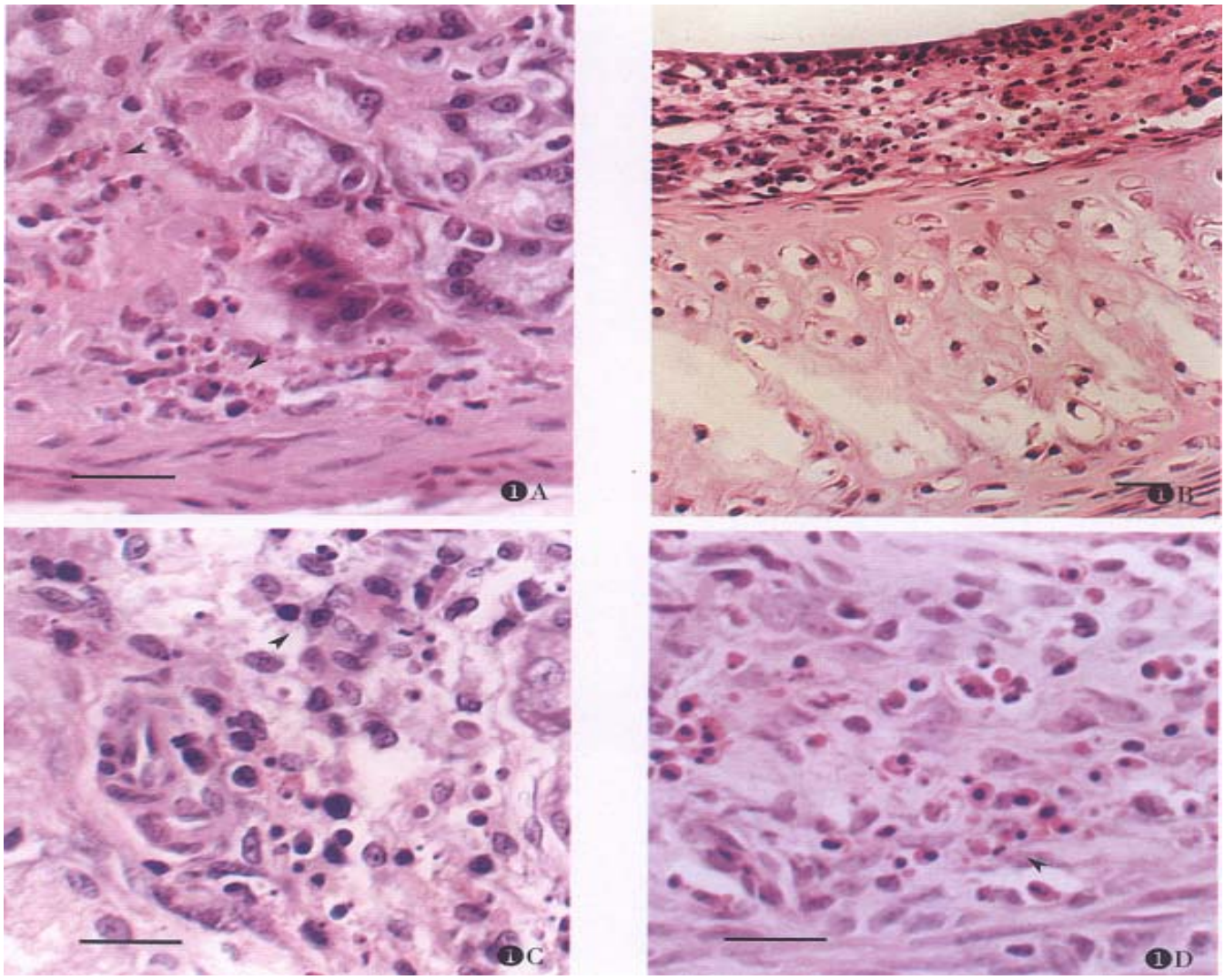

Figure 1 Occurrence of apoptosis in mucosa 3 hours after injection of CHX (3.0 mg · kg^{-1}) (bar = 20.0 μm, H&E). (A) Stomach apoptotic cells (bodies) lie in the lamina propria (arrows) of the mucosa. The parietal cells, chief cells and enterochromaffin-like cells seem not to be affected by treatment of CHX. (D) and (C) apoptosis in lamina propria of uterus and lacrimal gland. The apoptotic cells (bodies) were phagocytised by macrophages. (B) Mucosa apoptosis in trachea.

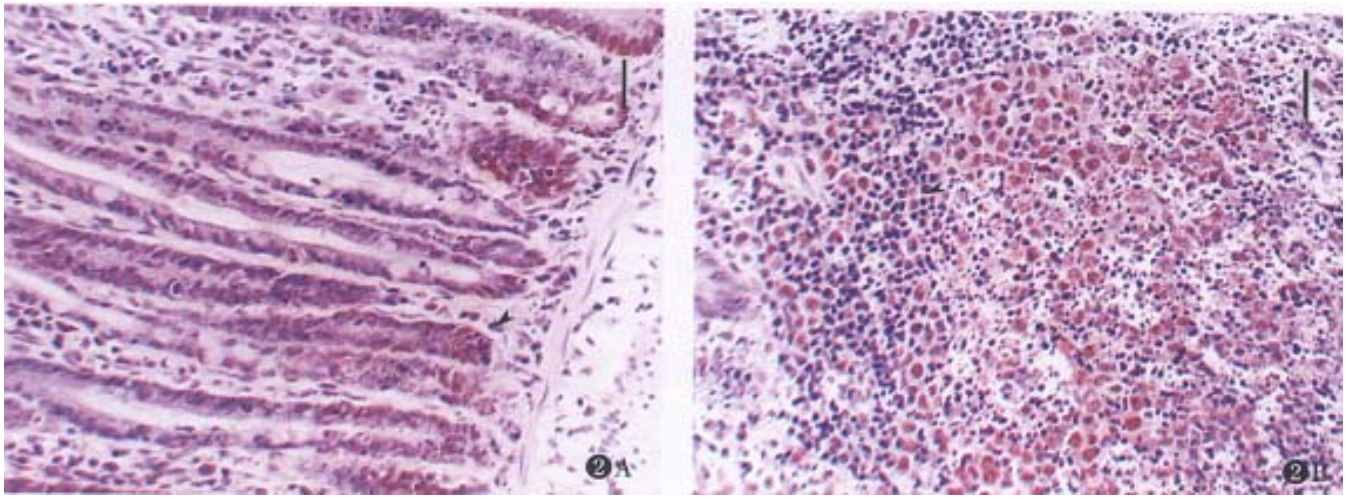

Figure 2 Expression of PCNA in mucosa after treated with CHX (3.0 mg · kg^{-1}) in 3 hours. (bar = 20.0 μm, stained with hematoxylin). (A) Colon. The apoptosis of crypt epithelial cells on the basement of mucosa were seen with expression of PCNA (arrow). (B) Peyer's patches in ileum. Apoptotic cells around the germinal center with expression of PCNA. The lymphocytes in parafollicular T-cell zone were morphologically normal and were negatively expressed with PCNA (arrow).

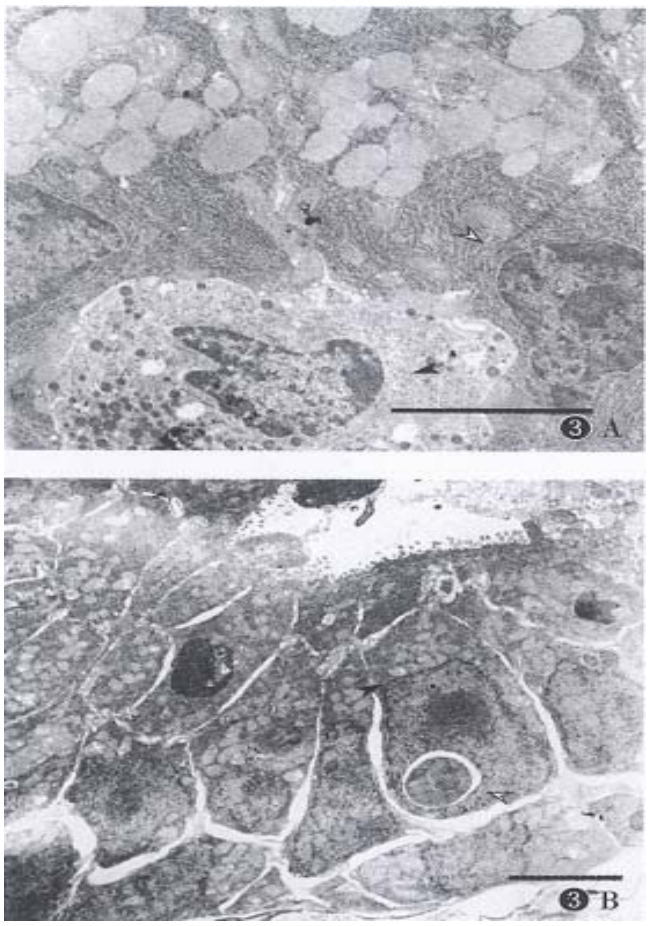

Figure 3 Morphological changes of cells after given CHX (3.0 mg· kg^{-1}) 3 hours later (bar = 4.0 μm). (A) Stomach. Normal appearance of chief cell (hollow arrow) and ECL cell (black arrow). (B) Jejunum. Condensed nucleus (hollow arrow) and proliferated mitochondria (black arrow).

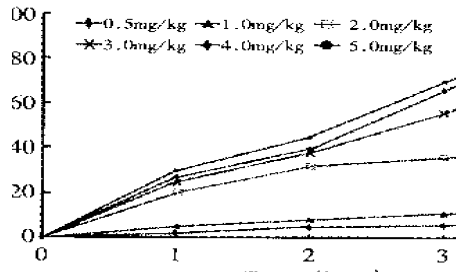

Figure 4 The time- and dosage-dependent effect of CHX on apoptotic bodies (cells) formation in rat uterus.

To estimate the effects of CHX on the induced apoptosis, apoptosis incidence could be calculated by counting apoptotic bodies and apoptotic cells of lamina propria in uterus. The incidence of apoptosis was dose-dependent (Figure 4). CHX 1.5 mg·kg^{-1} was nonlethal, and only inhibited the protein synthesis by 98% to 90% 1 - 3 hours after treatment[8]. Initial intraperitoneal injection of CHX at a dosage of 1.0 mg · kg^{-1} showed insignificant effects on apoptosis induction. Apoptosis was achieved significantly in 2h and reached its maximum 4h after a relatively large dosage of >2.0 mg·kg^{-1} body weight. In this study, the mortality rate of the rats was 33.3% (2/6) with 5.0 mg·kg^{-1}, and up to 83.3%(5/6) with 10.0 mg·kg^{-1} 18 h after CHX. CHX, when given intravenously or intraperitoneally, had the same effects on incidence of apoptosis.

DISCUSSION

In this study, mucosal lymphocytes in the immune system and a small portion of mucosal epithelial cells of the digestive and genital tracts could be induced to apoptosis by protein synthesis inhibitor CHX in rats. The apoptosis identified by morphologic features under light and electron-microscopy, could exhibit obviously in 2 to 4 hours after the rats were treated with relatively high dosage (>1.0 mg·kg^{-1}) of CHX, no cell necrosis or inflammatory changes were observed.

Lymphocytes primed by antigens in intestinal mucosal lymphoid follicles may transit through lymphatics and mesenteric lymph nodes, enter into circulation, and subsequently "homing" back to the intestinal lamina propria and other mucosal sites of respiratory tract, the female genital tract[1,2]. Peyer's patches are inductive sites and lamina propria and IEL are effector sites. These lymphoid tissues contain more immunocytes than any other tissues of the body[1,2]. Peyer's patches can be divided into three distinct regions: the specialized dome epithelium, the B-cell zone, and the parafollicular T-cell zone. In our experiment, most of lymphocytes in lamina propria and in the germinal centers of Peyer's patches underwent apoptosis,together with induction of IEL apoptosis in the digestive tract. Only a few lymphocytes in the parafollicular T cell zone of Peyer's patches were triggered to death by apoptosis. PCNA was expressed in the apoptotic cells in Peyer's patches and lamina propria PCNA. It has been demonstrated that elevated PCNA appear in the nucleus during late G1 phase immediately before the onset of DNA synthesis, and peaked during S phase[9]. These indicate that most of the apoptotic cells induced by CHX in common immune system were activated B and T lymphocytes (at least IEL in gut mucosa)[1,2,10,11].

Epithelial cells are constantly exfoliating to lumen and replaced by cells migrating from the proli-ferating zone, and homeostasis of this process is thought to be controlled by apoptosis[6]. In our study, CHX induced crypt epithelial cells of proli-ferating zone in the intestines and in the genital tract to apoptosis. These apoptotic cells were positively expressed with PCNA. Recent studies showed that passage through S phase is required for apoptosis, and S phase is the critical period to determine cell fate either to replicate or to die[12]. It seems likely that inhibition of protein synthesis accelerates the process of cell death by inhibition of *de novo* micromolecular synthesis[13,14].

Alternatively, inhibition of "protective protein" synthesis of cells initiated apoptosis. Paradoxically, a number of epithelial cells at proliferating stage, examined by light microscopy, were not triggered to apoptosis by CHX in this experiment, which is consistent with the aforementioned results of lymphocytes. There might be three explanations: one is that these "normal cells" appear "normal" at the early stage of apoptosis, the second one is that the propensity of these cells to undergo apoptosis are cell-cycle-phase directed, suggesting that CHX may only interfere with a certain phase of proliferating cell cycle, and the third explanation is that the phenotype of apoptosis is not critically dependent on protein synthesis and the endonuclease involved in DNA fragmentation is already in place[15].

Although lymphocytes and epithelial cells underwent apoptosis after rats were given CHX, the monocytes/macrophages in common immune system were not affected. It has been observed that increased macrophages actively moved to and appeared in tissues where apoptotic cells and apoptotic bodies were formed in clusters. They phagocytosed the apoptotic cells and apoptotic bodies. This phenomenon renders the apoptosis distinctly different from necrosis and easily recognizable in tissue slides. In addition, before the DNA fragmentation and formation of apoptotic bodies, electronmicroscopy showed that the morphologic changes of the cells in apoptosis would take place in chronological order separating from their neighbors, increasing number of mitochondria, condensed nucleus and cytoplasm. It suggested that the increased mitochondria within cytoplasm can be an early hallmark of apoptosis. Cytoplasm of apoptotic cells was strongly stained with eosin (Figure 1B). It indicates that proliferating mitochrodria may be protective/ compensatory to apoptosis in consideration of some "protective protein" like bcl-2 protooncogen protein synthesized in mitochondria[16].

It is reported that CHX can induce apoptosis in hepatic cells[8], intestinal crypt cell of proliferating cell types *in vivo*[17]. The former represents the first in vivo evidence of apoptosis induced by CHX in a quiescent organ. However, our findings indicated that CHX mainly affected activated lymphocytes and epithelial cells of proliferating phase, and we failed to observe that quiescent epithelial cells and endocrine cells succumb to apoptosis by CHX. Therefore, susceptibility to apoptosis is dependent on cell types. In case of stomach the parietal cell, chief cell and ECL cell were morphologically normal (Figure 1A, Figure 3A), and some parietal cells were found in mitosis phase. Proliferating cells were frequently encountered in the isthmus region of stomach[18]. Although these negative findings were observed in our study, it is conceivable these proliferating cells may undergo apoptosis induced by CHX.

In summary, CHX injection can severely damage the body immune system of rats, especially the mucosal immune system, but it influences the mucosal epithelial and endocrine cells only slightly though injury of epithelial cells may damage the integrity of epithelium at the early stage (unpublished data). In fact injection of CHX ($2.0\ mg \cdot kg^{-1}$) could cause diarrhea in rats for 3 days possibly due to enterobacterial infection, or it might be correlated with sIgA deficiency and malabsorption[19]. We also used this model to study mucosal lymphocytes involved in the mechanisms of gastric acid production (unpublished data). Therefore, this model will be useful in investigating the role of mucosal immune system in the pathogenesis of related diseases such as IBD, celiac diseases, and Sjögren' s syndrome, as well as turnover mechanism of mucosal immunocytes.

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Commentary

Current concept of Spleen-Stomach theory and Spleen deficiency syndrome in TCM

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Subject headings spleen deficiency syndrome; spleen-stomach theory

INTRODUCTION

Spleen-Stomach theory is an important constituent of the theoretical basis of Traditional Chinese Medicine (TCM), the Spleen here is not synonymous with the Spleen in western medicine anatomically, physiologically or pathophysiologically. Conceptually, Spleen-Stomach theory is a comprehensive one, it mainly involves the digestive system, its vegetative nervous system, immunologic function, hemopoiesis, muscle metabolism, endocrine function (thyroid, adrenal cortex and medulla), hepatic metabolic function, protein, nucleotide, energy, water and salt metabolism. Experimental researches on animal models and clinical studies on Spleen deficiency syndrome have yielded fruitful results in this field which lead to a better understanding of its mechanism and help open a new avenue for treatment of diseases relevant to Spleen deficiency.

Physiology of Spleen-Stomach[1]

The Spleen-Stomach has various physiologic functions as follows: Spleen governs transport and transformation. Spleen-Stomach transforms food into nutrients which are the sources of Qi and blood. The nutrients include glucose, amino acids, lipid, cations-anions and trace elements. Stomach is considered a reservoir which empties into the intestine. Water and salt absorption depends also on the transportating function of Spleen-Stomach. Qi and blood are both vital to life, Qi means energy, blood points to circulating blood and its formed elements, blood and nutrients furnish nourishment to all organs and tissues of the body.

The up- and down-bearing function of Spleen-Stomach

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Stomach governs down-bearing function and Spleen governs up-bearing which signify the motility, secretory, assimilative, absorptive and dispersing functions of upper digestrive tract, among which, gut hormones and functional activities of vegetative nervous system of the gut are involved. Dysfunction of up- and down-bearing function of Spleen-Stomach can cause gastrointestinal disturbances and various Spleen deficiency syndromes; furthermore, they may also affect the functions of other organs.

Spleen governs the flesh or muscles

Transport and transformation function of Spleen-Stomach provided nutrients for muscle and energy metabolism. When Spleen-Stomach is diseased, muscles will be atrophied and become asthenic.

Spleen governs thought

Brain activities depend normally on furnishing the nutrients, Qi (energy), blood, Yin and Yang. With adequate Qi and blood and normal function of Spleen-Stomach, brain activities can proceed normally.

Spleen manages blood

Spleen has hemopoietic functions; the blood encased within the vessels of all solid and hollow organs is managed by Spleen.

Relationship between Spleen-Stomach and other solid and hollow organs

Spleen and Heart The heart governs blood vessels and blood circulation. The lung is related to oxygen uptake. Dispersion of nutrients over the whole body depends on heart, lung and brain function which can also affect Spleen-Stomach motility and function.

Spleen and Kidney In TCM, the Kidney stores the body's essence, and its function also depends on the nutrients transformed by Spleen-Stomach. The transport and transformation function of Spleen-Stomach is also influenced by warming and nourishing functions of Kidney. The two are interrelated and mutually potentiating. The Kidney in TCM involves the genital-urinary system, sexual glands and hormones, immunologic function, nervous system and the heart in terms of western medicine. Spleen and Kidney are both

involved in water and salt metabolism. The Kidney-Yang denotes the hypothalamic-pitutary-adrenal, thyroid and gonadal axes, secretion of the corresponding hormones, sodium pump activity of red blood cells, caloric energy production and immunomodulating functions. The Kidney-Yin involves cAMP/cGMP activity.

Diagnostic approach and preventive treatment of Spleen-Stomach diseases[2]

Spleen-Stomach theory forms the basis of diagnostic approach and treatment of Spleen-Stomach disease, according to the presence or absence of Stomach-Qi. Poor appetite signifies grave prognosis and absence of Stomach-Qi; good appetite signifies good prognosis and presence of Stomach-Qi. By looking at the complexion and feeling the strength of pulse, one can predict the prognosis of the patient. Sallow face is the usual manifestation of Spleen disease. Color of skin over thenar muscles reflects Cold or Heat. Cyanotic hue of vein over thenar region indicates Cold in the stomach, whereas redness shows Heat in the stomach. During convalescence from postoperative state or infectious diseases, doctors should prescribe those herbal medicine for replenishing and modulating the Spleen-Stomach function.

Spleen deficiency syndrome is a multi-system and multi-organal functional impairment, but mainly manifests as digestive tract disturbance. It can be classified into Spleen-Qi deficiency,Spleen-Yang deficiency and Spleen-Yin deficiency. The diagnostic criteria in common are poor appetite, abdominal fullness after meal, loose bowel movements, pale or sallow complexion. The characteristic features of Spleen-Qi deficiency are fatigue, asthenia, atrophied muscle, pale tongue with thin white coating and moderate, weak pulse. Spleen-Yang deficiency is characterized by cold limbs, fear of cold, puffy pale tongue with slippery coating and slow fine pulse. Spleen-Stomach-Yin deficiency has dry mouth, low urine output, dry stool, shrunken smooth bare, red tongue and rapid fine pulse.

Pathophysiology of Spleen deficiency[1]

Clinical and animal studies were conducted with controls and statistical analysis made in the past years.

Changes in secretory and absorption functions of gastrointestinal tract

Salivary flow and salivary amylase activity. Salivary gland activity is related to Spleen function. In patients with chronic gastritis, gastric or duodenal ulcer or chronic colitis with Spleen deficiency syndrome, the salivary flow of parotid glands on citric acid stimulation decreased together with decrease in amylase activity. After Spleen-fortifying treatment, the condition was improved and normalized.

Gastrin and acid secretion. Serum gastrin level was found significantly lowered in Spleen deficiency syndrome but elevated after treatment. In peptic ulcer patients with Spleen deficiency, BAO and MAO were both significantly higher than normal. In chronic gastritis patients with Spleen deficiency, the day and night and 24h uropepsin activities were much decreased.

Pancreatic exocrine function, BT-PABA test. In peptic ulcer patients with Spleen deficiency, urine excretion of BT-PABA diminished. On treatment with Spleen-fortifying drugs, urine BT-PABA and amylase activity in chronic atrophic gastritis patients were increased.

Xylose excretion test. Decrease in xylose excretion in chronic atrophic gastritis patients with Spleen-deficiency syndrome indicated impaired absorption function of small intestine. After treatment, the xylose excretion rate was increased.

Motilin and gastrointestinal motility. In patients after major surgery on digestive tract with Spleen deficiency, serum motilin level was significantly higher than normal. The gastric tone was decreased with retention of fasting gastric juice but transit time of small and large intestine was reduced, resulting in rapid emptying, these motility disturbance led to loss of appetite and epigastric fullness after meal.

Structural and biochemical changes in gastric mucosa. In Spleen deficiency syndrome patients, the turnover rate of epithelial cells was hastened, showing a short life span. In the mucosal lamina propria of superficial gastritis with Spleen deficiency, the glandular atrophy was more severe, intestinal metaplasia more frequent, whereas in disharmonic Liver and Stomach type, the metaplasia was modest. Ultrastructural studies revealed reduced microvilli of epithelial cells, increased junctional width, membrane damage, swollen mitochondria with disrupted cristae and dilated endoplasm in parietal cells[4] decreased pepsinogen granules within chief cells; increased plasma cell infiltration in lammia propria. These changes were not seen in disharmonic Liver and Stomach type. Substance P and VIP were found increased in sigmoid colon, correlated with loose bowel movement. Elevated cAMP levels in gastric mucosa and plasma of Spleen deficiency syndrome were found, and plasma cAMP/cGMP ratio was decreased markedly in those patients with intestinal metaplasia. Likewise, gastric mucosal SOD content and plasma LPO also decreased significantly; these might correlate with metaplasia.

Dysfunction of vegetative nervous system of GI tract

Cerebral cortical function is extensively suppressed, presented with unstable somatic evoked potential, diminished amplitude and poor reproducibility. The hypofunction of sympathetic nervous activity

manifested a decrease in skin electric potential activity, reactivity of peripheral vessels to cold, urine VMA contentas well as plasma dopamine hydroxylase level; all of them increased after adequate treatment. In these patients, blood acetylcholine level was elevated, usually accompanied by bradycardia and lower systolic and diastolic blood pressure; these indicated presence of relative hyperfunction of parasympathetic nervous activity.

Motility and secretion modulating functions of GI tract are relevant to gut hormones. Besides, they are also related to modulating function of the vegetative nervous system of GI tract. Deficiency of Spleen in chronic diarrhea and peptic ulcer patients have overactive parasympathetic nervous system in the majority and hyperactivity of both parasympathetic and sympathetic nervous system in a minority of the patients.

Immunologic functional changes

In Spleen deficiency patients, peripheral blood lymphocyte count is lower than normal. In patients with chronic hepatitis B with liver depression and Spleen deficiency, it is also lower than normal, but can be restored to near normal after Spleen-fortifying therapy. T cell subset study revealed significantly decreased total T cell and TH lymphocytes. Among cancer patients with Spleen deficiency, CD4 was lower than normal, whereas CD_8 had no change. On treatment with astragalus and atractylodes, CD4 was elevated significantly.

Immunoglobulin G changed very little but the content of secretory IgA in GI disease with Spleen deficiency decreased significantly. After treatment with the Si Jun Zi decoction, these can be restored to the level of controls. Some Spleen-fortifying prescriptions can enhance proliferation of mice splenic cells and increase significantly the mice specific antibody secretory cell number, antigen-induced delayed allergic reaction and mixed lymphocytic reaction; they also enhance cytotoxic action of lymphocytes and promotes ConA-stimulated mice splenic cells to secrete IL-2. Phagocytic function of monocyte-macrophages also increase as seen by the clearance of carbon particles. In cancer-bearing patients, NK activity and TK activity are lowered but can be restored by Spleen-fortifying therapy. With Hp infection, these patients have a weaker lymphocyte and plasma cell infiltration and local SIgA response. It was believed that red blood cells also have the function of clearing circulatory immune complex and phagocytosis. Astragalus and Si Jun Zi decoction can restore the immunologic function of red blood cells, probably through their promoting effect on C_3b receptor expression and the activity on the red cells surface.

Recently, it was found that Spleen-deficiency patients had a high frequency of HLA-B_{12}-whereas disharmony of Liver and Stomach patients had a high frequency of HLA-B15. These showed that immune response is closely related with vulnerability to disease.

Endocrine changes

Urine 17-ketosteroid was found significantly lower than normal but there was no significant change in 17-hydroxysteroids as compared with normals. In patients with Spleen deficiency the level of catecholamine was also low. There was thyroid hypofunction in Spleen deficiency patients, with total T_3 and fT_3 significantly lower than normals, whereas rT_3 was significantly higher; low metabolic rate, low skin temperature, poor tolerance to cold and lack of adaptation to enviromental changes were also present. This poor tolerance to cold is a special feature of Spleen-Yang deficiency. Asthenia and loss of weight might also be due to hypofunction of thyroid.

Changes of fecal bacteria flora and *Helicobacter pylori*

In Spleen deficiency mice *Lactobacillus bifidus* and other lactobacilli were decreased but could be restored to normal after the Si Jun Zi decoction treatment. The enterobacteria pathogens were increased but could be reduced after treatment and *Helicobacter pylori* was also decreased in amount. Spleen-fortifying therapy lowers the rate of detection and amount of Hp[3].

Changes of trace elements in blood

In chronic hepatic disease with Spleen-deficiency the blood Zn was significantly lower than normal, but Cu was on the reverse. Zn is important in enzymatic action, nucleic acid synthesis, membranous function of red blood cells, hemopoiesis and cell respiration. In Spleen-Yang deficiency serum Mg was increased, whereas in Spleen-Yin deficiency it was extremely low. Fe was elevated in Spleen Qi deficiency, and Spleen-Yang deficiency patient at the age of 50 to 60 years, and ten years after it was elevated. In Spleen-Qi deficiency Mn and Cr were both increased significantly[4].

Muscle metabolism

The muscle glycogen and CPK activity in the quadriceps and plasma were all decreased significantly. The resulting asthenia, and muscular weakness were primarily due to energy depletion from lowered hepatic and muscle glycogen content. Besides, ATP, ADP and AMP contents were also much lowered, and LDH and succinyl dehydrogenase activities significantly elevated because of anaerobic glycolysis; those ions relevant to muscle contraction were decreased[5].

Serum total free amino acids and essential

amino acids including branch chained amino acids were all decreased; lysine, valine, glycine, threonine, tryptophan, isoleucine, serine, alanine and histone were all lower than normal. These may all contribute to muscle emaciation.

Treatment of Spleen-deficiency syndrome[1,6]

Many GI diseases, such as chronic atrophic gastritis, chronic pancreatitis with diarrhea, inflammatory bowel disease, coeliac disease may present Spleen-deficiency at certain stage of the disease. Because they have similar pathophysiology, they can be treated by the same principle but with emphasis on different aspects.

The Si Jun Zi decoction is the major Spleen-fortifying therapy for Spleen-deficiency syndrome, including Ginsen or Codonopsis, Atractylodes, Poria, Glycyrrhiza. It promotes absorption and dispersion of nutrients to the whole body, increases physical strength and mental activity, and has multiple effects on the digestive organs, immunity, hemopoiesis, blood circulation, hepatic synthetic function, muscle metabolism, etc. By adding Astragalus , the immunologic function and small intestinal muscular tone can also be increased.

Motility and absorptive function of GI tract

The Si Jun Zi decoction inhibits the small intestinal activity, presented with decrease of amplitude and spasmodic contraction of small intestine. It possesses anticholinergic effect as well as antihistaminic response. The inhibitory effect on intestinal tract is both neurogenic and myogenic. It also has modulating effect on the function of vegetative nervous system. In Spleen deficiency and Qi deficiency, there are inhibition of transport and sodium pump on the epithelial cells which can be restored by the Si Jun Zi decoction. Codonopsis-Poria-Atractylodes powder can promote absorption of water and chloride ion so as to improve the diarrhea of Spleen deficiency.

Immunologic function

The Si Jun Zi decoction promotes cellular immunity, enhances peritoneal macrophagic phagocytosis, corrects the immunosupressive effect of cyclophosphamide and cortricosteroids, and reduces the inhibitory effect on bone marrow and atrophy of thymus, Spleen by cyclophosphamide. Experimental study revealed that abundant extramedullary erythroblasts and active proliferation of lymphoblast control the atrophic effect of cyclophophamide in adrenal contex and testes, also decreases the toxic adverse effect of chemotherapeutic drugs. It also increases the humoral immunity by increasing antibody production for infected patients with Spleen deficiency, it ameliorates and restores the weight loss of thymus in undernourished mice as well as the structure within the thymus such as the thickness of cortex/medulla ratio and the diameter of thymus cell nucleus to normal.

Hemopoietic action

Ginsen promotes marrow cell DNA and protein synthesis, and codonopsis promotes red blood cell formation. If ferrous sulfate is given concomitantly, restoration can be hastened.

Hepatic synthetic function

It can increase plasma albumin, hepatic RNA and glycogen contents in favor of tissue repair and hepatic detoxification.

Effect on skeletal muscle

In Spleen-Qi deficiency, the mitochondria decreases in number with decrease of oxidase, and increase of anaerobic glycolysis. The Si Jun Zi decoction can restore muscle glycogen, lipid, ATP synthesis and the ultrastructures of mitochondria to near normal; food consumed, body weight, physical strength and mental spirit all improved.

Blood circulation of gastric wall and mesentery

On the basis of the Si Jun Zi decoction, the addition of *Astragalus*, *Cinnamon Twig*, *Saussure*), *Tangerine Peel*, may dilate arterioles, increase cabillary blood flow and tissue perfusion, and promote tissue repair.

Action of individual component of the Si Jun Zi decoction:

① Ginsen, small dose stimulates central nervous system and large dose inhibitsit. Ginsen increases mental reactivity and the contents of dopamine, nor-epinephrine in the brain, promotes brain RNA and protein synthesis, increases blood supply, and blood oxygen to the brain; all these are the pharmacologic basis for increasing brain activity. Besides, it has anti-fatigue action and increases tolerance to cold. Ginsen enhances both cellular and humoral immunity, also activates the phagocytic function of macrophages and activity of NK cells, and in addition, it also induces interferon formation.

In the endocrine system, it enhances the hypothalamic-pituitary-adrenal cortical, thyroid and gonadal axis function. It increases cardiac contractility by inhibiting the sodium pump on cardiac muscle cell membrane. It promotes release of insulin and elevates blood insulin level, enhances the synthesis of protein, RNA and DNA, lowers blood lipid, and improves hepatocytic function. As a therapeutic drug for Spleen deficiency, emphasis should be laid on its systemic effect.

② Codonopsis, it also enhances immunologic function, both cellular and humoral immunity, and increases response to stress and

tolerability to hypoxemia. Codonopsis is important for Spleen-fortifying. It can antagonize the decrease in gastric mucosal content of prostaglandin E_2 and aminohe-xose induced by ind omethacin and aspirin, and inhibits acid secretion, hence being useful for combating ulceration and mucosal damage. It can also modulate GI motility, and has bidirectional effect on intestinal motility. It can decrease blood viscosity by inhibiting platelet aggregation, and also has some vasodilating action. Furthermore, it has varying degrees of inhibition on the cyclo-oxygenase pathway, TXA_2 synthetase; and these are dose dependent.

③Atractylodes, being a representative of Spleen-fortifying drug, it modulates intestinal motility bidirectionally, increases smooth muscle tone of GI tract, improves the white slippery coating of tongue, and cellular and humoral immunity and protein synthesis. Moreover, it acts on the hypothalamic-putuitary-adrenal cortical axis.

④Poria, trelaxes smooth muscle of GI tract, diminishes the contraction amplitude, reduces acid secretion, and has anti-necrosis and anti-degenerative actions on hepatocytes in hepatitis. Its polysaccharide enhances cellular immunity, increases the weight of thymus and lymph node, and hence is used in treatment of cancer of GI tract.

⑤ Glycyrrhyza, having acidity lowering and antipasmodic actions, it diminishes the smooth muscle tone of the intestine, amplitude of contraction, and inhibits ileal contraction induced by acetylcholine. It also has anti-inflammatory and anti-allergic effects. It can increase the cytochrome P-450 content of hepatocytes, and detoxification effect. Besides, it has corticosteroidal effect.

⑥Astragalus, when added to the Si Jun Zi decoction, it can potentiate the immuno-enhancing effect, including induction of interferon production and increase in antibody formation. Astragalus also increases cardiac contractility, in particular, the ejection fraction, promotes blood cells formation and maturation, restores the reticulocytes and megakareocytes to normal. Through activation of phosphorylase mediated by cAMP, it can promote mitosis, differentiation and growth of marrow cells. Furthermore, it increases the RNA, DNA and protein synthesis of the liver. It has an antioxidant action. Moreover, it may increase corticoid secretion and elevates the plasma level of cortisol. It has long been used in treatment of atrophic gastritis, peptic ulcer and other gastrointestinal diseases with Spleen deficiency by increasing cAMP content for gastric mucosal repair. It is also used in chronic hepatitis with Spleen deficiency to increase hepatocytic RNA, DNA and protein synthesis.

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Clinical and experimental study of therapeutic effect of Weixibaonizhuan pills on gastric precancerous lesions *

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Subject headings Weixibaonizhuan pills; stomach neoplasm/TCM therapy; precancerous conditions; gastric mucosa; Chinese herbal

Abstract

AIMS To observe the therapeutic effect of Weixibaonizhuan pills (胃细胞逆转丸) on gastric precancerous lesions.

METHODS Thirty patients with gastric precancerous lesions were treated with Weixibaonizhuan pills for 3 months. Of the 36 cases, 13 (36.1%) were mild atrophic gastritis, 14 (38.9%) moderate atrophic gastritis and 9 (25.0%) severe atrophic gastritis; among them 22 (61.1%) and 27 cases (75.0%) were accompanied with intestinal metaplasia (IM) and dysplasia (DYS) respectively. Of the 36 patients, 20 were men and 16 women, aged from 30-60 years and those aged 30-59 years accounted for 61.1%. The course of disease ranged from 3 months to 21 years, and 20 (55.6%) of them had a course of 5 - 10 years. The clinical manifestations were fullness of the abdomen (31 cases),abdominalgia (27 cases), anorexia (30 cases), gas eructation (26 cases), acid regurgitation (6 cases) and loose stool (9 cases). When treatment ended, the improvement of patients' clinical symptoms, atrophy of gastric mucosa, IM and DYS were analysed.

RESULTS After 3 months' treatment with Weixibaonizhuan pills,7 cases recovered, 11 cases were much improved, 13 cases showed some improvement, and 5 cases were ineffective; the total rate of symptomatic improvement was 86.1%. Of the 13 cases with mild atrophic gastritis, 11 cases changed into superficial gastritis, and 2 cases had no changes. Of the 14 cases of moderate atrophic gastritis, 4 cases changed into superficial gastritis, 7 cases changed into mild atrophic gastritis, and 3 cases had no changes. Five of 9 cases of severe atrophic gastritis were reduced to moderate atrophic gastritis, and 4 cases had no changes. The total effective rate was 77.8% in chronic atrophic gastritis. Of the 9 cases with mild IM, IM disappeared in 6 cases and 3 showed no change. Of the 10 cases with moderate IM, it disappeared in 2 cases, 5 cases changed to mild IM, and 3 cases had no change. One of the 4 cases of severe IM changed to moderate IM and 3 had no change. The total effective rate was 63.6% in IM. Of the 16 cases of mild DYS, 11 cases showed disappearance of DYS and 5 had no change. In 9 cases of moderate DYS, 2 showed disappearance, 5 changed to mild DYS and 2 had no change. Two cases of severe DYS, both showed no change. The total effective rate was 66.7% in DYS. Before treatment, the I, II, III and IV degree positive expressions of CEA were present in 13, 12, 9 and 2 cases, respectively, whereas after treatment, the positive expressions were present in 25, 7, 3 and 1, respectively. Before treatment, the I, II, III and IV degree positive expressions of PCNA were present in 16, 11, 10 and 4 respectively, but after treatment, they were present in 21, 9, 5 and 1 respectively. In short, the positive expressions of CEA and PCNA of gastric mucosa were significantly decreased after treatment ($P<0.01$).

CONCLUSION Weixibaonizhuan pill has a therapeutic effect in gastric precancerous lesions.

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INTRODUCTION

Gastric carcinogenesis often arises from some precancerous lesion for cancer development. Effective treatment of such lesions would be helpful in decreasing the risk of gastric cancer. We have used Weixibaonizhuan pills (WXBNZ pills 胃细胞逆转丸) to treat them and have obtained a good result.

PATIENTS AND METHODS

Patients

Thirty-six patients with gastric precancerous lesions were diagnosed through gastroscopy and mucosal biopsy[1]. Among them, 13 (36.1%) cases were mild chronic atrophic gastritis (CAG), 14 (25.0%) cases moderate CAG, and 9 severe CAG. Twenty-

two (61.1%) and 27 (75.0%) cases were accompanied intestinal metaplasia (IM) and dysplasia (DYS), respectively. Of the 36 patients 20 were men and 16 women; their age ranged from 30 - 60 years, and those between 40 - 59 years of age accounted for 61.1%. The course of disease ranged from 3 months to 21 years, and 20 of them (55.6%) had a course of 5 - 10 years. The clinical manifestations were as follows: ① abdominal fullness or feeling of obstruction in the stomach (31 cases), worse in afternoon or after eating and abated after eructation or passing wind from bowels; ② abdominalgia (27 cases) in middle abdomen and epigastrium, more often vague, or distending, burning, stabbing, with tenderness; ③ anorexia (30 cases); ④eructation after eating or of frequent occurrence (26 cases); ⑤acid regurgitation (6 cases) became severe when fasting; and ⑥loose stool (9 cases), watery or unformed.

Methods

All patients took WXBNZ pill (composed of 15 kinds of herbs including *Radix Codonopsis pilosulae, Radix Salviae Miltiorhizae, Herba Solani Nigri, Radix et Rhizoma Rhei, Rhizoma coptidis* , etc, prepared by the Research and Treatment Center of Gastroenterology of Taiyuan) 6 grams three times a day for a course of 3 months. Gastroscopic and pathologic studies of biopsy specimens were performed before and within 2 weeks after treatment. During gastroscopy, 1 biopsy specimen was taken each from the antral lesser curvature, greater curvature, anterior wall and antrum-body transitional zone, respectively, and additional 1-2 biopsy spe-cimens were taken from the grossly identifiable lesions. The examinations were made by fixed doctors. In addition to routine HE staining, we also used immunohistochemical technique to detect the expression of CEA and PCNA of gastric mucosa. CEA antibody (rabbit against human) and PCNA antibody (mouse against human) were provided by Zymed Co. and universe type biotinylated secondary antibody was made in our lab. The staining procedure was performed according to the literature[2].

RESULT

Improvement of clinical symptoms after treatment

Of the 36 cases, 7 recovered with a disappearance of clinical symptoms, 11 had significant improvement of symptoms, 13 showed some improvement, and 5 had no change. The total effective rate was 86.1%.

Improvement of gastric mucosal atrophy

In 13 cases of mild CAG, 11 cases changed to superficial gastritis, and 2 had no change, the effective rate being 84.6%. Among 14 cases of moderate CAG, 4 changed to superficial gastritis, 7 changed to mild CAG (Figures 1, 2), 3 cases had no change, the effective rate being 78.6%. Five of the 9 cases of severe CAG changed to moderate CAG (Figures 3, 4), and 4 cases had no change, the effective rate being 55.6%. The total effective rate for CAG was 77.8%, and the therapeutic effectiveness seemed to be better in mild and moderate CAG.

Improvement of IM after treatment

In the 9 cases of mild IM, it disappeared in 6 cases, and 3 cases had no change, the effective rate being 66.7%. In the 10 cases of moderate IM, 2 cases showed a disappearance of IM, 5 cases changed to mild IM and 3 cases had no change (Figures 5, 6), the effective rate being 70.0%. Only one of the 4 cases of severe IM changed to moderate IM and 3 had no change, the effective rate being 25.0%. The total effective rate for IM was 63.6% and the therapeutic effectiveness was better in mild and moderate IM.

Improvement of DYS after treatment

In the 16 cases of mild DYS, 11 cases showed its disappearance and 5 had no change, the effective rate being 68.8%. In the 9 cases of moderate DYS, 2 had a disappearance of DYS, 5 changed to mild DYS and 2 had no change, the efficacy rate was 77.8%. Both of the 2 cases of severe DYS showed no change after treatment, the efficacy rate was 0.0%. The total effective rate was 66.7% in DYS, and the therapeutic effectiveness was better in mild and moderate DYS.

Improvement of gastric CEA

A semiquantitative method was used to judge the brown-stained positive cells[3]. The positive degree was scored according to the number of positive cells. Grade I: 0% - 25%, grade II: 26% - 50%, grade III: 51% - 75%, and grade IV: 76% - 100%. The expression of CEA decreased significantly after treatment ($P<0.01$), (Table 1).

Table 1 Positive expression of CEA in gastric mucosa before and after treatment with WXBNZ pills

Treatment	Grades			
	I	II	III	IV
Before	13	12	9	2
After	25	7	3	1

$u = 27.68$, $P<0.01$.

Improvement of gastric PCNA

The positive criteria of PCNA was similar to CEA. The PCNA expression decreased significantly after treatment ($P<0.01$) (Table 2).

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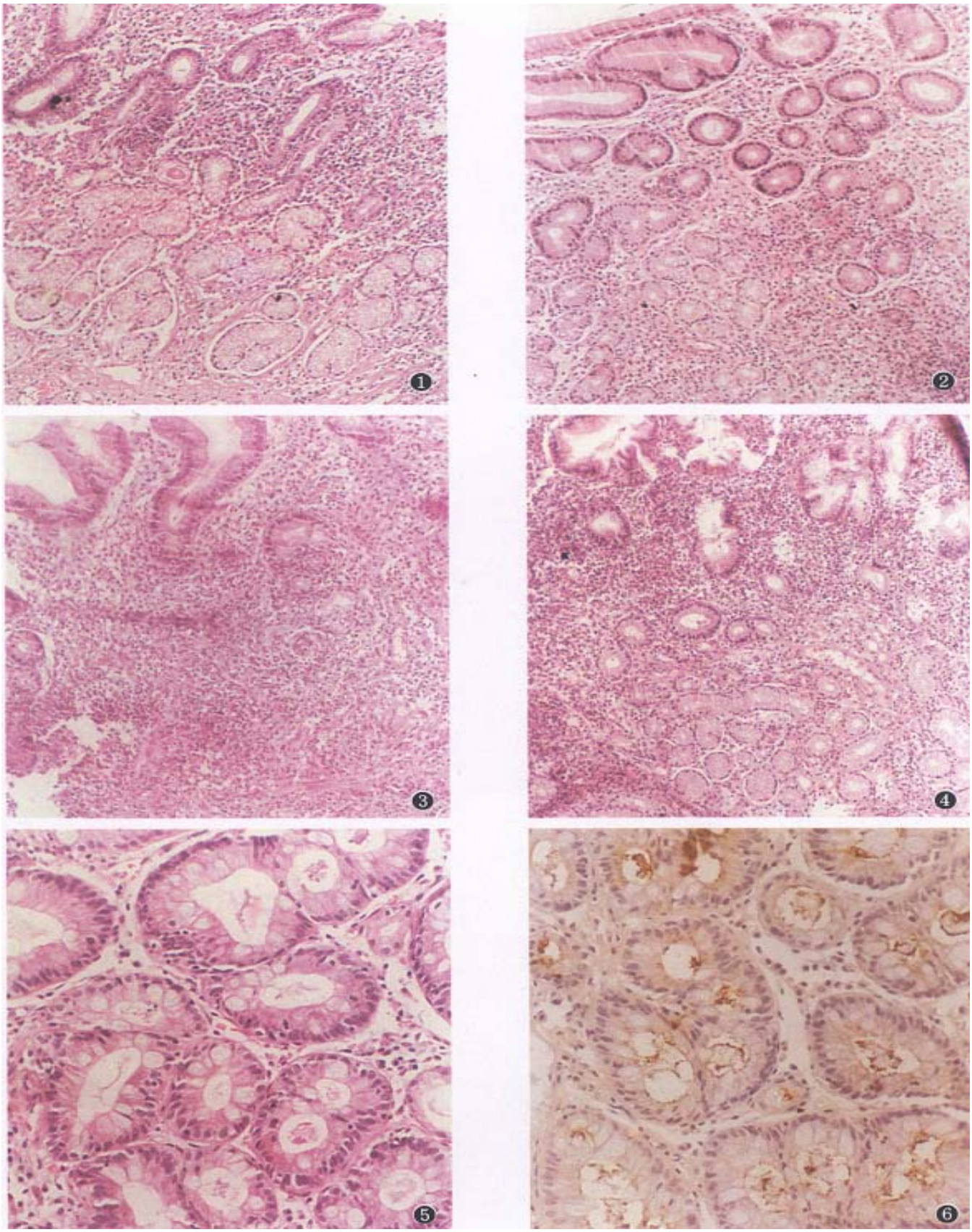

Figure 1 Moderate CAG before treatment. (HE×100)
Figure 2 Mild CAG after treatment. (HE×100)
Figure 3 Severe CAG before treatment. (HE×100)
Figure 4 Moderate CAG after treatment. (HE×100)
Figure 5 Severe CAG accompanied with severe IM, there is no significantly difference between before and after treatment (HE×200)
Figure 6 Severe CAG accompanied with severe IM, there is nosignificantly difference of CEA expression between before

and after treatment (LSAB×200)

Table 2 Positive expression of PCNA in gastric mucosa before and after treatment with WXBNZ pills

Treatment	Grades			
	I	II	III	IV
Before	26	11	10	4
After	21	9	5	1

u = 13.85, P<0.01.

DISCUSSION

The rate of cancer transformation in patients with CAG was reported to be 0.2%[4]; those with mild DYS was 2.5% - 11%[5]; moderate DYS, 4% - 35%[5,6]; severe DYS, 10% - 83%[7]; and IM, 1.9%[4]. It can be suggested that treatment of procancerous lesions may decrease the incidence of gastric cancer effectively. The long-standing chronic gastritis may result in gastric mucosal atrophy, dysplasia and IM of glandular epithelium. As a consequence of such pathologic changes, the patient may have hyposecretion of gastric acid and pepsin, along with symptoms of dyspepsia, such as anorexia and abdominal distention after eating, loose stool, malnutrition, etc. According to the theory of traditional Chinese medicine (TCM), such manifestations belong to an " insufficiency of the Spleen and Stomach *qi*" . Another group of patients presenting symptoms of epigastric pain, eructation, acid regurgitation, nausea, etc belong to "disharmony between the Liver and the Stomach" with stagnation and reversed flow of *qi* , together with the production of Heat. So chronic gastritis with precancerous lesions is a complicated ailment in TCM with regard to its cause ad pathogenesis. The rationale of its management is to strengthen the Spleen and Stomach, to enhance the body resistance, to consolidate the constitution, to promote flow of *qi* and blood, and to disperse Heat. Only by this way can the symptoms and pathology be improved effectively. We used WXBNZ pills, which was composed of 15 kinds of herbs and had the above-montioned effects of strengthening the Spleen and Stomach, strengthening the body resistance, to consolidate the constitution, promoting flow of *qi* and blood and dispersing Heat, in treating the patients with gastric precancerous lesions. The total effective rates for clinical symptoms, CAG, DYS, IM were 86.1%, 77.8%, 66.7%, 63.6%, respectively. These results indicated that WXBNZ pills had a good therapeutic effect in gastric precancerous lesions.

From the results in this study we can infer that WXBNZ pills has a better therapeutic effects in mild and moderate CAG, DYS and IM, but gives poor results in severe CAG, DYS and IM. Therefore, early diagnosis and early treatment of such lesions are recommended. In normal gastric mucosa, CEA staining is negative or restricted to the secretory margin of cells. In precancerous lesions, the CEA staining showed a positive expression not only at the secretory margin of cells but also in the apical cytoplasm or further dispersed in the whole cell membrane and cytoplasm. We found in our previous study that positive expression of CEA increased progressively as the precancerous lesions progressed[2]. The positive expression of CEA may reflect the beginning of a deviation or transition of normal state of cell differentiation toward malignancy. In this study, the positive expression of CEA decreased and disappeared gradually after WXBNZ pills treatment, indicating that WXBNZ pill might have some effect of inhibiting the CEA expression and accele-rating the recovery of gastric mucosal function. Recent studies have shown that PCNA is related intimately to the DNA synthetic phase (S phase), and is a good marker reflecting the activity of cell proliferation[3]. In this study, the high positive expression of PCNA in the gastric mucosa with precancerous lesions decreased distinctly after WXBNZ pills treatment. These results suggested that WXBNZ pills could inhibit the cell proliferative activities and abnormal hyperplasia of gastric mucosa in patients with precancerous lesions.

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Aflatoxin sufferer and p53 gene mutation in hepatocellular carcinoma *

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Subject headings Aflatoxin B1; genes, p53; mutation; carcinoma, hepatocellular; liver neoplasms

Abstract

AIM To study the p53 gene mutation and its relationship to aflatoxin B1 exposure in hepatocellular carcinoma (HCC).

METHODS Restriction fragment length polymorphism analysis method was used in 62 HCC samples, and DNA direct sequencing in another 45 HCC samples.

RESULTS In HCC and AFB1 high and low-risk areas, 36/52 (69%) and 2/10 (20%) cases were found losing the HaeIII allele respectively, suggesting one of the base G mutation at the p53 gene codon 249. Similar results appeared in DNA direct sequencing, 20/35 (57%) and 1/10 (10%) respectively mutated at the codon 249 third base G to C transversion.

CONCLUSION In HCC after AFB1 exposure, mutation of p53 gene is fixed at codon 249 third base and take the form of G to T transversion. This is a definite marker of mutation which is induced by AFB1 mutagen. It is applicable for molecular epidemiologic survey of the sufferers of AFB1 among HCC cases and for discovering more unknown natural AFB1 contaminated areas.

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INTRODUCTION

Previous reports from Qidong and southern Africa discovered that the p53 gene of hepatocellular carcinoma (HCC) had a mutational hot-spot at codon 249[1-3]. Later there were repeated reports from Qidong. However, reports from worldwide other than those two areas showed no such special mutational hot-spot in HCC[4-6]. It was supposed that codon 249 mutation might be caused by exposure to aflatoxin B1 (AFB1) which was a common enviromental factor prevalent in the two mentioned areas. Aspergillus flavus is widely distributed throughout the world. However, well-known natural high AFB1 contaminated areas are uncommon. It is of significance to study one more known natural high AFB1 risk area Fusui and its neighboring counties where HCC mortality rate was high up to 40/100 000 annually. AFB1 exposure and hepatitis B virus (HBV) were both suspected to be the principal aetiological factors.

MATERIALS AND METHODS

Surgical specimens of HCC were fixed in formalin and embedded in paraffin. The pathological diagnosis was made by standard histologic criteria. The DNA of 62 HCC samples were amplified focus on the p53 gene exon 7 by polymerase chain reaction (PCR). Primers were introduced by Murakami *et al*. PCR products containing exon 7 were digested with the restriction enzyme Hae III, then eletrophoresed for restriction fragment length polymorphism (RFLP) analysis. Another 45 HCC samples were sent for DNA direct sequencing. In the meantime, all samples were stained for HBsAg by ABC method of immunohistochemistry.

RESULTS

A lot of HCC samples came from high HCC prevalent and high AFB1 exposed area in Guangxi lost the Hae III restriction site GG/CC by method of RFLP analysis. It suggested a mutation occuring at the sequence AGG, CCC. Undoubtedly, the mutational point occurred at the hot-spot codon 249 nucleotides G rather than non-hot-spot C of codon 250. Surprisingly, this mutation was found in 36/52 samples (69%). Compared to the samples from a low prevalent area in Guangxi, the frequency was 2/10 (20%). The difference was significant (P<0.01). The results by direct DNA sequencing were similar. The samples from high prevalent area were 20/35 (57%) mutation at codon 249 third

nucleotide G to T transversion, while low prevalent area showed this transversion only in 1/10(10%). The difference was also significant ($P<0.01$) . The total positive rate of HBsAg was 94.4%, with no difference between high and low prevalent areas.

DISCUSSION

The p53 tumor suppressor gene is one of the genes with great interest, because it is commonly mutated in human cancer, and the spectra of p53 mutations in these cancers provide clues to the etiology and molecular pathogenesis of tumors. In HCC, p53 gene mutation is related to AFB1 and HBx protein of HBV. The present data is a research on the spot on great number of cases. The conclusions are as follows: the principal etiology and molecular pathogenesis of HCC at the part of high prevalent area in Guangxi are caused by AFB1 without doubt, and HBV chronic infection is also high. AFB1 induces p53 gene mutational hot-spot in the high prevalent local area up to 57% - 69%. This hot-spot is located at exon 7 codon 249 third nucleotide G to T transversion. It is a special and stable mutational point. It is useful for HCC molecular epidemiologic study for general survey whether the residents are at the risk of aflatoxin exposure, and distinguish the AFB1 sufferers of HCC cases individually. Until now, there has been no report from high AFB1 contaminated area where HCC samples escape this p53 gene mutational hot-spot. More and more human materials from high AFB1 areas show the mutation point clustering at the codon 249 third nucleotide rather than the first or second nucleotide, and only G to T single form but not G to A or C multiple forms mutation. Therefore it would be confirmed as a mutational marker of a AFB1 sufferer in human HCC.

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Effect of garlic and garlic-green tea mixture on serum lipids in MNNG-induced experimental gastric carcinoma and precancerous lesion*

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Subject headings garlic tea; stomach neoplasms/prevention & control; precancerous conditions/blood; lipids/blood

INTRODUCTION

To study effect of garlic and garlic-green tea mixture on serum contents of Tch, LDL and HDL in MNNG-induced gastric carcinoma (GC) and precancerous lesion (PL) in Wistar rats.

METHODS

Serum contents of Tch, LDL and HDL in normal control group (n = 10, NG), MNNG group (n=30, MG), prevention group (n = 30, PG), treatment group I (n = 20, TG I) and treatment group II (n = 20, TGII) were detected by PGE 6000/COD.

RESULTS

Serum Tch and LDL of rats of MG (6.86 ± 1.39, 3.72 ± 1.10) and its GC(6.95 ± 1.37, 3.77 ± 1.08) and PL(6.42 ± 1.04, 3.56 ± 0.74) were lower than that of NG (8.74 ± 1.89, 5.89 ± 1.61),PG(7.73 ± 3.18,4.96 ± 2.89) and its GC(8.36 ± 3.41, 5.93 ± 3.31) and PL (7.45 ± 3.16,4.55 ± 2.71), TGI (8.86 ± 1.75, 5.38 ± 1.76) and its GC (9.10 ± 2.27, 5.55±2.51) and PL (8.61 ± 1.17, 5.22 ± 0.55) and TGII (8.16 ± 0.76, 5.32± 0.72) aod its GC(8.52 ± 0.67,5.96 ± 0.48) and PL (8.02 ± 0.79,5.09 ± 0.65), respectively ($P<0.01$-0.05). Serum HDL of MG rats (2.76 ± 0.48) and its GC(2.79 ± 0.48) were remarkably higher than that of MG (2.20 ± 0.85) and GC of PG (2.24 ± 0.38) ($P<0.05$).

CONCLUSION

Experimental gastric carcinoma and precancerous lesion were associated with hypocholesterolaemia, LDL and HDL. Garlic and garlic-green tea mixture can inhibit and reverse MNNG-induced gastric carcinoma and precancerous lesion in Wistar rats.

nucleotide G to T transversion, while low prevalent area showed this transversion only in 1/10(10%). The difference was also significant (P<0.01) . The total positive rate of HBsAg was 94.4%, with no difference between high and low prevalent areas.

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Serum Tch and LDL of rats of MG (6.86 ± 1.39, 3.72 ± 1.10) and its GC(6.95 ± 1.37, 3.77 ± 1.08) and PL(6.42 ± 1.04, 3.56 ± 0.74) were lower than that of NG (8.74 ± 1.89, 5.89 ± 1.61),PG(7.73 ± 3.18,4.96 ± 2.89) and its GC(8.36 ± 3.41, 5.93 ± 3.31) and PL (7.45 ± 3.16,4.55 ± 2.71), TGI (8.86 ± 1.75, 5.38 ± 1.76) and its GC (9.10 ± 2.27, 5.55±2.51) and PL (8.61 ± 1.17, 5.22 ± 0.55) and TGII (8.16 ± 0.76, 5.32± 0.72) aod its GC(8.52 ± 0.67,5.96 ± 0.48) and PL (8.02 ± 0.79,5.09 ± 0.65), respectively (P<0.01-0.05). Serum HDL of MG rats (2.76 ± 0.48) and its GC(2.79 ± 0.48) were remarkably higher than that of MG (2.20 ± 0.85) and GC of PG (2.24 ± 0.38) (P<0.05).

CONCLUSION

Experimental gastric carcinoma and precancerous lesion were associated with hypocholesterolaemia, LDL and HDL. Garlic and garlic-green tea mixture can inhibit and reverse MNNG-induced gastric carcinoma and precancerous lesion in Wistar rats.

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CEA and AFP expression in human hepatoma cells transfected with antisense IGF-I gene *

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Subject headings insulin-like growth factor-I; carcinoembryonic antigen; α-fetoprotein; carcinoma, hepatocellular; tumor cells, cultured; radioimmunoassay

Abstract

AIM To determine whether antisense insulin-like growth factor-I (IGF-I) gene can modulate CEA and AFP expression in human hepatoma cells (HepG2).

METHODS Transfection of HepG2 cells was accomplished using Lipofectin reagent. Northern blot analysis confirmed the antisense IGF-I RNA of the transfected cells. CEA and AFP levels were measured using radioimmunoassay.

RESULTS Human hepatoma cell lines (HepG2) were transfected with antisense IGF-I gene. Northern blot analysis confirmed that antisense IGF-I RNA was expressed in the transfected cells. The effect of antisense IGF-I gene on CEA and AFP expression was demonstrated by the fact that the CEA and AFP levels in the supernatant of transfected cell culture were significantly lower as compared with the parent cells, [CEA 7.0 μg/L ± 0.76 μg/L and 3.29μg/L ± 1.80 μg/L ($P < 0.05$) and AFP 53.63 μg/L ± 6.02 μg/L and 9.0 μg/L ± 5.26 μg/L ($P < 0.01$), respectively].

CONCLUSION The malignant potentiality of the transfected cells was partially suppressed.Antisense IGF-I gene can modulate the expression of CEA and AFP in human hepatoma cell lines (HepG2)

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INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a cytokine with multiple biological functions, which can stimulate cell differentiation and DNA synthesis and transcription[1]. In recent years, it has been found that many kinds of tumors expressed abnormal IGF-I, e.g., hepatoma, astrocytomas, etc. IGF-I might be important mitogens in the growth of many tumors[2]. Tumor cells may be able to enhance their own growth by synthesis of endogenous IGF-I. This process of autocrine secretion contributes to the partialautonomy and rapid growth in tumor cells[2-4]. We have applied the antisense strategy to block the function (e. g., by binding to a splice junction) of IGF-I in tumor cells so as to promote its degradation, and alter the structure of the target sequence. In a recent report, the stable transfectants of rat glioblastoma cells transfected with antisense IGF-I gene lost its tumorigenicity, and the inhibition of IGF-I expression elicited a highly immunogenic phenotype in glioma cells[3,4]. On the other hand, tumor markers related with the malignant hepatocarcinoma are CEA and AFP. IGF-I expression was increased significantly in the cell proliferation and high malignancy of human hepatoma. It can be used in the subsidiary diagnosis of the relapse and the metastasis of cancer, and in the research of neoplastic transformation and primary tumor diagnoses. To observe the effect of antisense IGF-I gene on the tumor markers (CEA and AFP), antisense IGF-I gene was transfected into human hepatoma cells (HepG2) to observe whether antisense IGF-I gene can regulate the changes of CEA and AFP, and the effect of neoplastic transformation.

MATERIAL AND METHODS

Cell lines and materials

Human hepatoma cells were purchased from the Institute of Shanghai Cell Biology. Antisense IGF-I expression plasmid was obtained from the University of Case Western Researve. Hygromycin was purchased from Sigma. Lipofectin and RPMI-1640 were purchased from GIBCO. The DIG system for hybridization was purchased from Boehringer Mannheim Biochemica. The CEA and AFP kit were the products of Institute of Shanghai Biologic - Products.

The antisense IGF-I plasmid preparation and its DNA separation, appraisal and purification

Detailed methodology followed Kliieg Ler M[5] and Trojan *et al*[3]'s.

Antisense IGF-I gene transfer into tumor cells mediated by Lipofectin

Transfection of HepG2 cells was accomplished using Lipofectin reagent (GIBCO) according to the instructions. Solution A: 100 μl RPMI-1640 containing 10 μg/L antisense IGF-I gene. Solution B: 90 μl RPMI-1640 containing 10 μl Lipofectin. Both solutions were mixed gently, and placed at room temperature for 15min. Two serum-free RPMI-1640 was added to each tube containing the Lipofectin reagent-DNA complexes, and mixed gently and overlay out cells. The cells were incubated for 5h - 12h at 37℃ in a CO_2 incubator. Two ml of RPMI-1640 was supplemented with 10% FCS and the cells were incubated at 37℃, 5% CO_2 incubator for another 48h. The cells grow in the presence of hygromycin until positive clones were selected.

Northern blotting hybridization

Total RNA in cells was extracted according to Kliieg Ler M[5]. Transfer blot hybridization was carried out as described in manual of DIG system kit. A dilution series of the total RNA was transferred to Nylon membrane and baked for 30min at 120℃. The membranes were put in a sealed plastic bag and used for Northern hybridization

Measurement of CEA

CEA kit contained CEA marker, 125I-CEA, CEA antibody and immune separation reagent. The operation and analysis of the results were carried out according to the manual.

Measurement of AFP

AFP kit contained marker,125I-AFP, first antibody of AFP, analysis reagent of AFP and second antibody of AFP. The operation and analysis of the results were carried out according to the manual.

Statistical methods

Data were analyzed using the Student's *t* test.

RESULTS

The analysis of Northern Blot

The antisense IGF-I plasmid was purified by abstraction and electrophoresis. It was wrapped up by Lipofectin and conducted into human hepatoma cells (HepG2). IGF-I antisense transcripts were selected in the presence of hygromycin. The total RNA in positive clone was extracted and its RNA transcription levels were analyzed by Northern Blot hybridization (Figure 1). The results showed that strong expression of the antisense transcript and the IGF-I transcripts of parent HepG2 cells was not apparent.

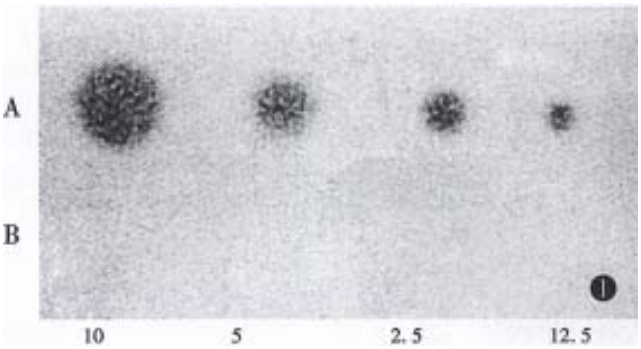
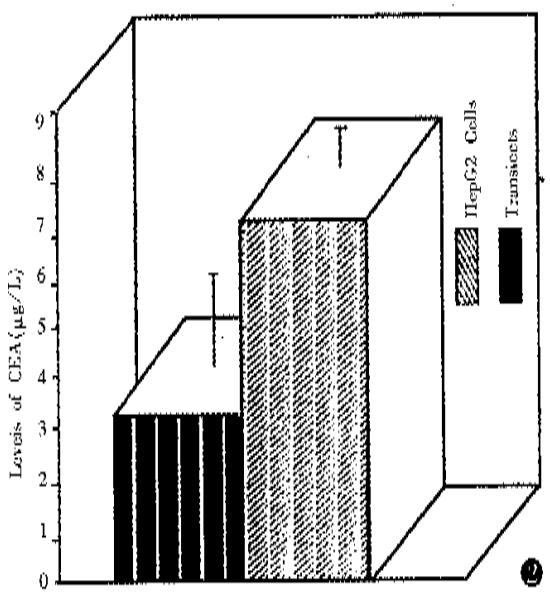


Figure 1 Northorn blot analysis of antisense IGF-I transcripts in human HepG2 cells. Lane A, RNA of transfectants cells, Lane B, RNA of parents cells.



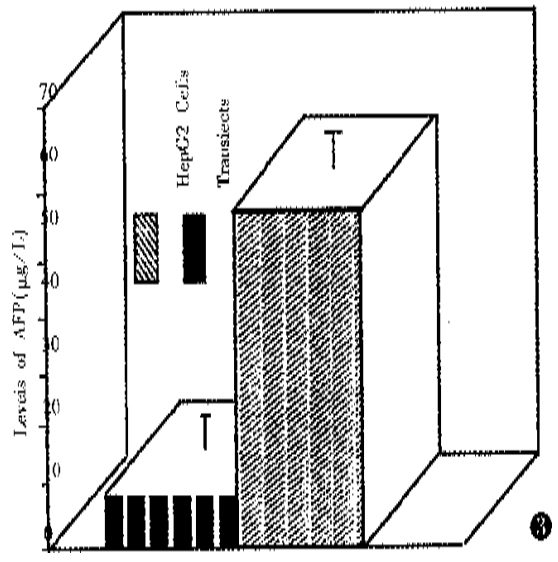


Figure 2 Expression of CEA in human HepG2 cells transfected with antisense IGF-I gene and parents HepG2 cells.

Figure 3 Expression of AFP in human HepG2 cells transfected with antisense IGF-I gene partents HepG2 cells.

CEA levels of HepG2 cells transfected with antisense IGF-I

The transfectant cells were kept in the presence of hygromycin for 8 - 12 days. After the transfectants and parent cells were cultured for 24 hours, the supernatants were collected and CEA levels were determined (Figure 2). The CEA levels of positive clones were markedly lower than that of the parent cells (7.0 μg/L ± 0.76 μg/L and 3.29 μg/L ± 1.80 μg/L, respectively) (P<0.05).

The levels of AFP of antisense IGF-I transfectants of HepG2

The supernatants were collected by the same method for CEA. AFP levels are shown in Figure 3. The AFP levels of positive clones were lowered markedly as compared with parent cells (53.63 μg/L ± 6.02 μg/L and 9.0 μg/L ± 5.26 μg/L, respectively) (P<0.01).

DISCUSSION

It has been known for a long time, abnormal materials related with the diseases existin the sera of cancer patients. The tumor markers are used for the diagnosis, prognosis and treatment of tumor. Although, the tumor markers were expressed nonspecifically by tumors and produced differently, they are still important indexes to predict carcinogenesis. CEA and AFP are carcinoembryonic protein. They appear in the embryonic term and disappear in adult. When they appear in the serum of adult, it shows that the growth of tumor was active so that these proteins were secreted into the blood. The half-life of CEA and AFP in plasma were 6 - 8 and 6.5 days. Recent reports indicated that CEA was a member of immunoglobulin supergene family[6]. Both were important tumor markers in the course of the carcinogenesis and development. As both were regarded as the index of the tumor biological changes and of the malignant degree in vivo, in this study, CEA and AFP were used as the index of positive clone biological changes after tumor was transfected by antisense IGF-I gene. It showed that antisense IGF-I gene affect the changes of tumor markers related with malignancy of the tumors. Because many gastrointestinal tumors were produced by CEA and AFP, human hepatoma cells (HepG2) were selected as the target tumors. Antisense IGF-I gene was wrapped up and transfected into the HepG2 cells, and cells grew in the presence of hygromycin for the positive clone. Northern Blots proved that transfectants of HepG2 cells showed detectable antisense IGF-I transcripts. The positive clone grew in the presence of hygromycin for 8 - 12 days. After the transfectants and parent cells were adjusted to 2×106/well for 24 hours the supernatants were collected. Figures 1-2 show the results of radioactive immune technique. The CEA and AFP levels of positive clones were markedly lower than that of the parent cells [CEA, 7.0 μg/L ± 0.76 μg/L and 3.29 μg/L ± 1.80 μg/L (P<0.05) and AFP, 53.63 μg/L ± 6.02 μg/L and 9.0 μg/L ± 5.26 μg/L (P < 0.01), respectively]. The results indicated that transfectants could affect the levels of CEA and AFP. When the secretive levels of both decreased, it suggested that malignancy of positive clone was lower than that of the parent cells and original biological courses were changed. At present, a lot of papers show that many kinds of tumors expressed abnormal IGF-I and suggest the involvement of the continuous proliferation and tumorigenic phenotype[2]. After antisense IGF-I gene was transfected into target cells, a specially nucleotide sequence was expressed in cells that was complementary to a portion of IGF-I gene in target cells. The process and transcript of IGF-I mRNA were inhibited. As the secretion of IGF-I in target cells were changed, it could affect the role of biology in cells, the event could inhibit the growth and the tumorigenicity of rat glioblastoma cells and murine teratocarcinoma in vivo[3,4]. That antisense IGF-I gene blocks the corresponding IGF sequences may be the reason for the decrease of CEA and AFP in target cells. The exact machanisms await further researches.

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Arterial chemoembolization for hepatocellular carcinoma *

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Subject headings Liver neoplasm/therapy; Lipiodol; sinobufagin microsphere; gelatin sponge; chemoembolization, therapeutic

Abstract

AIM To study the therapeutic effects of transcatheter arterial three-segment chemoembolization for hepatocellular carcinoma (HCC).

METHODS According to the anatomy of vessels, the tumor capillary networks, muscular arterioles and feeding arteries were successively occluded using lipiodol ultra-fluid (LUF), sinobufagin microsphere (SBMs) and particles of gelatin sponge (PGS). In this series of 80 cases, therapeutic effects were evaluated in 76 cases.

RESULTS There were 22 cases (28.9%) with partial response and 41 (53.9%) with minor response in the 76 evaluated patients. The 6-month, 1-year, 2-year and 3-year survival rates were 97.4%, 86.8%, 46.1% and 27.6% respectively.

CONCLUSION This regimen was a rational chemoembolization method for HCC patients.

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INTRODUCTION

Transcatheter arterial embolization was recommended for the treatment of unresectable HCC in the 70s. Recently, various embolic materials and anticancer agents have been developed. But these methods still have some unsolved problems such as the level of tumor vessels embolization, selection of embolic agents and anticancer drugs, etc. Therefore, we devised the three-segment chemoembolization of the tumor vassels (TSCTV) using lipiodol ultra-fluid, sinobufagin microsphere and particles of gelatin sponge for the treatment of HCC patients. The results of the clinical trials are reported below.

MATERIALS AND METHODS

Patients

Eighty consecutive patients with HCC were observed from March 1990 to March 1992. There were 72 males and 8 females, aged from 24 to 69 years (average 49 years). None of them underwent chemotherapy before. The diagnosis of HCC was established by various imaging techniques (including ultrasonography, US; computed tomography, CT; magnetic resonance imaging, MIR; and hepatic angiography, HAG;) and serum alpha-fetoprotein (AFP). The histologic diagnosis was made in 10 cases.

The cases were classified into four stages according to the Manual for Staging of Cancer[1], 2 cases as stage I (2.5%), 6 cases as Stage II (7.5%), 62 as Stage III (77.5%), and 10 as Stage IV (12.5%). The serum AFP levels were greater than 400μg/L in 42 cases (52.5%). Hepatitis B surface antigen was positive in 62 cases (77.5%). The characteristics of these patients are shown in Table 1.

Response criteria

The clinical response to the treatment was assessed objectively by the change in tumor size, which was estimated by US, CT and MRI before and after the therapy. The reduction in tumor size was measured at the same image level, presenting the maximum diameter. In cases of the multiple tumors, the largest mass was measured in the same way[2]. Response criteria were defined based on the reduction of the perpendicular diameter in the tumor as follows: ①A complete response was total disappearance of tumor; ②A partial response was a reduction in tumor size of more than 50%; ③ A minor response was a reduction of 25% to 50%; ④

No change was a change in tumor size less than ± 25%; ⑤Progressive disease was an enlargement of more than 25%. Response criteria were determined once a month for 6 months after TSCTV. Response criteria had to be maintained for at least a month.

Preparation of chemoembolization agents

SBMs were prepared in the department of pharmacy of our college. The drug microsphere, with a mean diameter of 200μm, was composed of approximately 6% (w/w) of sinobufagin in gelatin[3]. The doses of embolic and anticancer agents were determined by the tumor size and degree of liver dysfuction.

Usually, 10 ml - 20 ml LUF and 50 mg - 100 mg SBMs were used when the tumor diameter was less than 10 cm, and 20 ml - 30 ml and 100 mg - 200 mg respectively when the tumor size was more than 15cm. The doses of the anticancer drugs were aclarubicin (ACR) 50 mg and cir-diaminedichloroplatin (CDDP) 80 mg. For patients with poor liver function anti-cancer drug doses should be reduced. The anticancer drugs were divided into two portions of same dose. A portion was suspended in LUF to make emulsion, the other portion was mixed in 10 ml of radiopaque contrast medium and SBMs to form mixture. The PGS, with a mean diameter of 1mm, was prepared in our laboratory.

TSCTV

TSCTV was performed through the femoral artery using the technique of Seldinger. A 6.5-French (Cook Co., USA) angiographic catheter was inserted superselectively into the hepatic artery feeding the target tumor.

Initially, the emulsion of the LUF and anticancer drugs were slowly infused till the vessels within tumors were filled. Subsequently, the microsphere mixture was gently infused till the tumor arteries completely disappared. Finally, the PGS, approximatly 1 g - 2 g, were infused until the feeding artery of the tumor was occluded. All of these procedures were performed under fluoroscopic guidance to avoid the refluence or spill-over of the embolic agents and anticancer drugs. The second therapy was performed about a month after the first TSCTV. TSCTV was repeated at 3 - 8 months depending on the patients' condition. This therapy should be carried out immediately if any of the followings appeared: increase in tumor size, occurrence of new focus within the liver, inadequate accumulation of LUF in the tumor, reelevation of serum AFP (>200 μg/L) or GGT (r-Glutamyltranspeptidase>350μg/L).

RESULTS

TSCTV courses and drugs doses

A total of 240 courses of TSCTV were performed, averaging 3 courses per patient, with a range of 1 to 6 courses. Fifty-six cases (70%) underwent more than 3 courses. The mean dose of embolic agents and anticancer drugs in TSCTV are shown in Table 2.

Response

Of 80 patients, 4 was excluded from this study because surgical operations were performed about a month after TSCTV. The remaining 76 patients (95%) were evaluated for responses. According to the response criteria, 22 (28.9%) had partial response, 41 (53.9%) minor responses, while no change in 10 cases (13.1%) and progressive diseases were observed in 3 (3.9%) cases (Table 3). The state of the tumor reduction maintained 4.5 to 49 months. The liquefaction and necrosis within tumors occurred in 60 (79%) cases after the first TSCTV. The necrosis area ranged from 2.5 cm to 6.5 cm (mean 3.8 cm × 3.2 cm). The second hepatectomy was performed in 6 cases within 3 - 6 months after 1 - 3 courses of TSCTV. The focuses were resected in 5 (6.6%) of 6 cases. The histological examination demonstrated that there were massive coagulation necrosis and fibrosis of tumor tissue in all cases. No living tumor cells could be seen in the specimens in 2 cases (Figure 1).

Survival rate

The starting point was defined as the day 0 initial TSCTV therapy. The 6-month, one-year, 2-year and 3-year survival rates were 97.4% , 86.8%, 46.1% and 27.6% (Table 4). The longest survival time was up to 49 months. During the disease palliation phase, the life quality of the patients with tumors was satisfactory.

Table 1 Clinical characteristcs of patients with hepatocellular carcinoma

Average age in year (range)	49(24-69)
Sex (male: female)	72:8
Serum AFP level (μmol/L)	
<50	18(22.5%)
50-399	20(25%)
>400	42(52.5%)
HBsAg+	62(77.5%)
TBILT(mg/L)	16.9± 6.0
ALT(u/l)	68.9±36.6
ALB(g/L)	38.4± 4.4
Child's grade (a:b:c)	30:47:3
Stage (I:II:III:IV)	2:6:62:10

TBILT: bilirubin, ALT: glutamic-pyruvic transaminase, ALB: albumin.

Table 2 Mean doses of embolic agents and anticancer drugs

TSCTV courses	No of patients	Embolic agents		Anticancer drugs		
		SBMs (mg)	LUF (ml)	SB (mg)	ACR (mg)	CDDP (mg)
1	80	145.0	22.3	8.7	41.8	80.0
2	72	114.3	13.7	6.9	37.9	75.6
3	56	107.8	15.4	6.5	40.8	76.4
4	24	91.7	11.7	5.5	40.0	80.0
5-6	6	100.0	11.2	6.0	40.0	80.0
Mean	240	111.8	14.9	6.7	40.1	78.4

SB: Sinobufagin

Table 3 Response to TSCTV in evaluated patients

Stage	CR	PR	MR	NC	PD	Total
I	0	0	0	0	0	0
II	0	0	2	2	0	4
III	0	22	36	4	0	62
IV	0	0	3	4	3	19
Response	0	22	41	10	3	76
Percentage	(0%)	(28.9%)	(53.9%)	(13.1%)	(3.9%)	

Table 4 Survival of 76 patients undergoing TSCTV

Stage	No of patients	No of survival (year)			
		0.5	1	2	3
I	0	0	0	0	0
II	4	4	4	3	2
III	62	62	60	32	19
IV	10	8	2	0	0
Total	76	74	66	35	21
Percentage		(97.4%)	(86.8%)	(46.1%)	(27.6%)

Laboratory examinations

The liver function, peripheral white blood cell counts, platlet counts and AFP level were examined before and after TSCTV (1, 2 and 4 weeks). The serum AFP, which was more than 200 μg/L before treatment in 62 patients, apparently fell in 58 patients (94%) about 2 weeks after TSVCTV. Serum AFP was lowered to normal (AFP < 200 μg/L) after 2 - 4 courses of TSCTV in 28 (48.3%) cases. The changes of serum AFP level in 42 cases (AFP>400 μg/L) are shown in Figure 2. The ALT level showed dropping tendency a week after TSCTV and then, elevated again in some patients after 2 - 3 weeks. The peripheral WBC of all patients increased after first TSCTV, $<3.5\times10^9$/L in 2 cases after the third TSCTV. The results of laboratory examinations are shown in Figure 3.

Side effects and complications

The postembolization syndrome (including fever, right hypochondrium pain, and nausea and vomiting) was seen in all patients after TSCTV. The serious degree of the syndrome was strongly correlated with the area of the liquefaction and necrosis within tumor and the doses of the embolic agents and anticancer drugs. After the first TSCTV, the syndrome was severe in some patients and generally maintained 1 to 3 weeks. The side effects and complications of TSCTV are shown in Table 5.

Table 5 Side effects and complications

Side effects and complications	Incidence No.(%)
Fever 38-39℃	71(88.7)
>39℃	18(22.5)
Nausea and vomiting	60(75.0)
Right hypochondrium pain	72(90.0)
Cholecystitisa	5(6.2)
Liver abscess	1(1.2)
Hapatic failure	4(5.0)
Ascites	6(7.5)

[a]The floccule was found in gall bladder by US

Figure 1 The resected specimen showing massive coagulation necrosis and fibrosis in tumor.

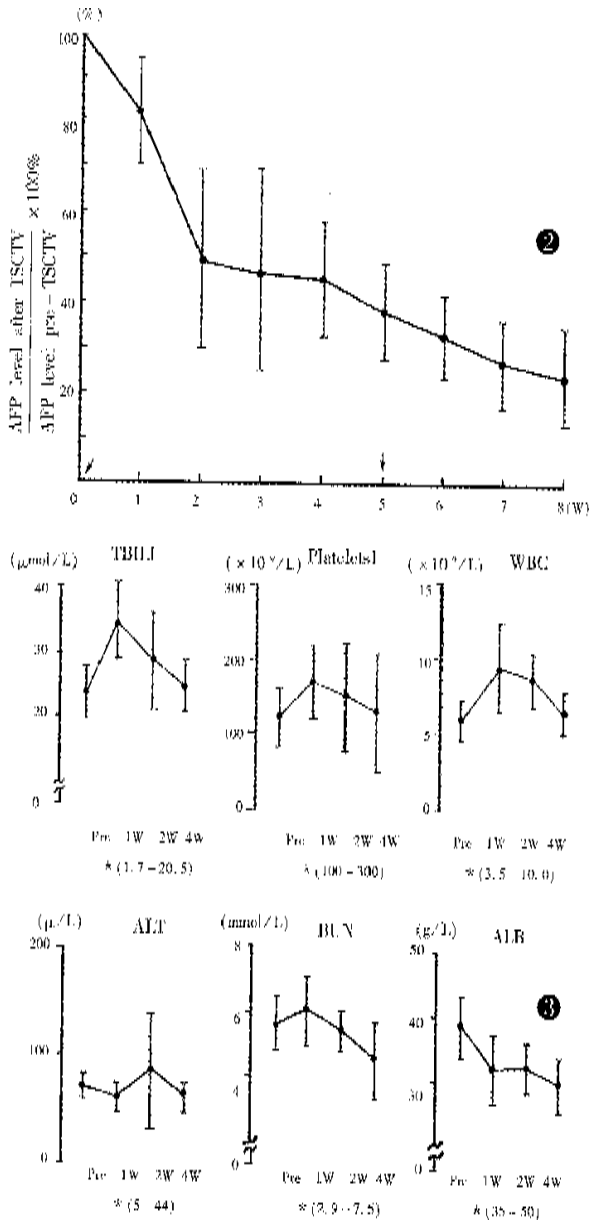

Figure 2 Serum AFP levels in 42 cases (AFP > 400μmol/L) after TSCTV, assuming that pre-embolization value was 100% (↓ TSCTV treatment).

Figure 3 Changes in blood chemical data after first TSCTV (pre: level before TSCTV; W: week; *: normal range)

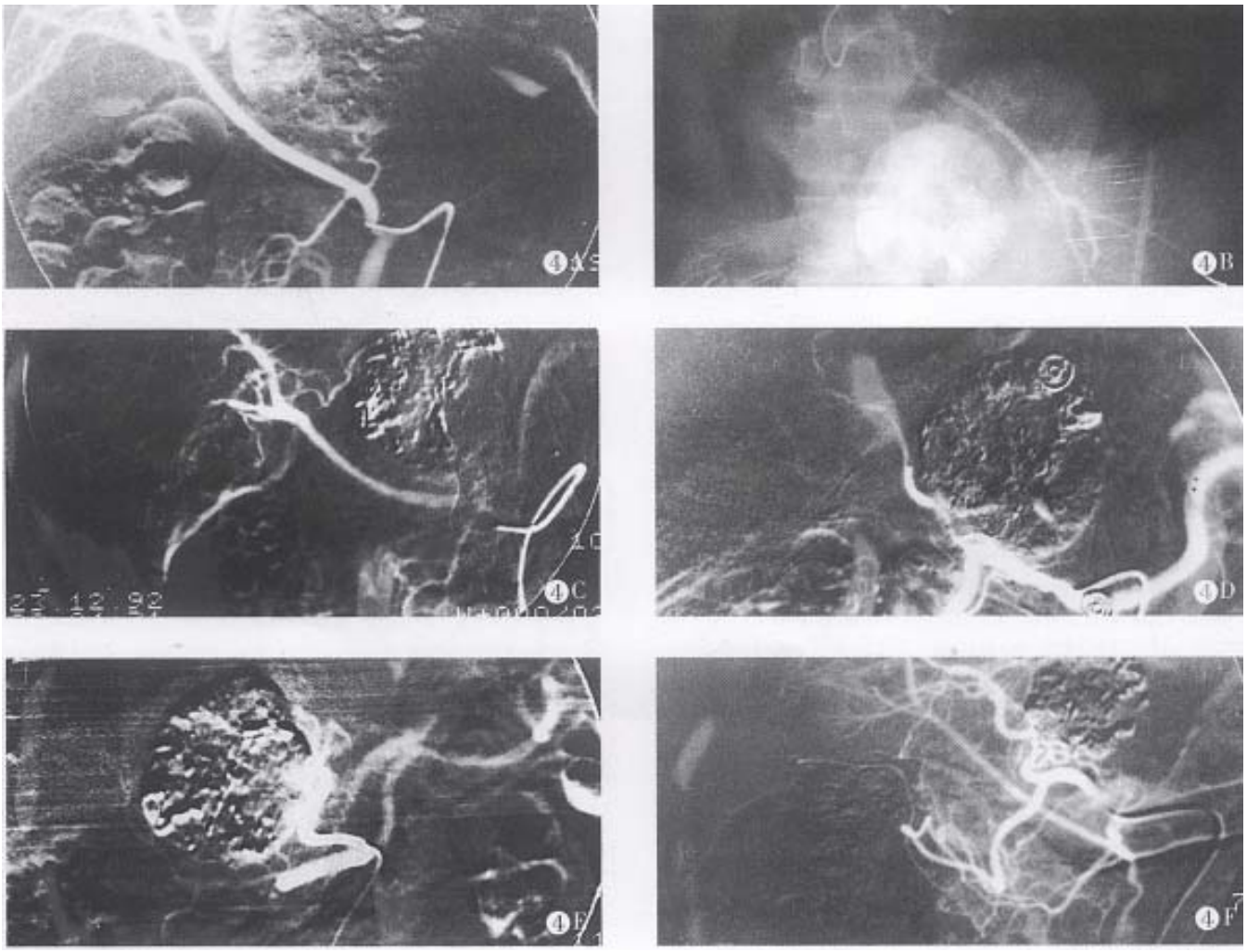

Figure 4 A. The hepatic right artery derived from superior mesenteric artery after TSCTV was performed. The left hepatic artery derived from gastroduodenal artery and supplying left and lower 1/3 of the tumor, to prevent embolic agents reflux, lipiodol chemoembolization (LCE) was performed.

B. The tumor vessels disappeared completely and the lipiodol accumulated within tumor after embolization.

C. The lipiodol was obviously washed out in the LCE area after 4 months, TSCTV and LCE were repeated.

D. The lipiodol was washed out fourth in the LCE area after 6 months.

E. LUF 5ml and SBMs 50mg were infused through hepatic left artery, the tumor vessels completely disappeared, the LUF accumulated in the LCE area.

F. The tumor obviously shrank (PR) and lipiodol accumulated within tumor after 19 months.

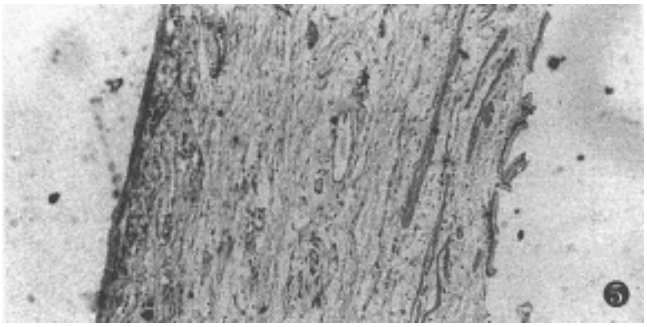

Figure 5 The necrosis of the gallbladded mucosa.

DISCUSSION

According to the electronic microscopic observation, the arterial system of the HCC is composed of three kinds of successive vessels. There are massive neonate capillary networks within tumor consisting of single layer endothelium cells. Its angiographic imaging is revealed as vascular mass. The successive vessels are muscular arterioles with diameters of about 100 μm - 250 μm. There are extensive arterio-portal vein shunts in these massive neonate vessels. The blood flow of the portal vein might reflux into the empty arterioles through these shunts as soon as the feeding arteries are occluded. The blood supply of the tumor renews in short time so that the inhibiting effect in tumors disappear soon. The successive feeding arteriols usually become wide and tortuous and are accompanied by “stealing blood”

phenonenon[4,5]. In light of the vessels anatomy, we designed the TSCTV method. There are several possible mechanisms of TSCTV that can prolong the survival rates: ①The capillary networks, muscular arterioles and feeding arteries of the tumor are successively occluded using three embolic agents of va-rious diameters and properties so that direct ischemic changes within tumors are caused by liquid embolic agent, LUF, and solid embolic agent, SBMs; while proper occlusion of feeding arteries surrouding the tumor is also encountered by expansive embolic agent, PGS. Thus, the occlusion of tumor vessels is more rational and complete. ②The less viscosity and better compatibility of LUF are favourable to occlusion of capillary networks in tumor and dispersion of anticancer drugs into oil phase[6]. ③By complex chemical cross linking, the degradation of the gelatin microsphere is delayed in vivo. After infusion, SBMs can block blood flow, delay the "washout" of LUF (Figure 4), prevent reflux of the portal vein blood and, simultaneously, slowly releave the drug locally. ④While occluding feeding and collateral arteries of the tumor, the PGS can avoid the refluxing of embolic agents and anticancer drugs into gallbladder and gastroduodenal arteries, resulting in severe side effects[7]. ⑤Because anticancer drugs suspended in LUF and SBMs, forming the structure of "three-ply-board" after infusion introduced as regional chemotherapy, anticancer drugs seem to be delivered to and accumulated selectively within the tumor[8,9]. ⑥ Sinobufacin, as an anticancer drug of Chinese traditional materials, has its own efficiency on HCC treatment, which is superior to fluorouracil. The drug can cause vessels inflammation and the remarkable endovasculitis and leading secondary embolization in the embolized vassels, enhancing the effect of arterial occlusion. In addition, the drug is very effective for cardiotonic, diuresis, antisepsis, analgesia and increased peripheral white blood cells, enhancing body immunofunction, etc[3]. These complementary pharmocologic effects can augment the therapeutic effects and reduce systemic side effects. ⑦ACR and CDDP are, at present, a better combination of chemotherapy, and have synergistic anticancer effects[7]. ⑧This method caused relatively mild, damage to liver and renal functions so that it is acceptable to most patients.

The outcomes of our clinical trial demonstrate that TSCTV can significantly improve the completeness of the tumor vassels occlusion, the area of li-quefaction and necrosis of the tumor, stay time of embolic agents within tumor vessels, partial response rate and survival rate, as compared with the treatment using microsphere or lipiodol alone.

It was found that after occlusion, the collateral circulation occurred sooner or later in most tumors. Where there was vessels occlusion, there was the formation of collateral circulation. Therefore, occlusion should be performed until the tumor vessel completely disappeared in each course of treatment so that the extensive and fast necrosis of the tumor cells can be induced. The necrosis of tumor cells might result in high response rates, survival rates and prevent metastasis within the liver[3,7]. The doses of embolic agents should be modest, as excessive embolic agents might cause reflux and side effects.

Tumor progression occurred in some patients with time. Timely re-TSCTV could usually inhibit or prevent the tumor progression. Therefore, repeated TSCTV was very important in ensuring the-rapeutic effects. We found that more than 3 couses of TSCTV was essential.

However, the effect of TSCTV was not so satisfactory in stage IV patients, especially in those with tumor thrombus of the major portal bronchus. In our study, there were 4 cases of hepatic failure. Therefore, this method should be carefully adopted in those patients.

The post-embolization syndrome was significant by this method. The causes might be as follows: ① Complete occlusion of the tumor vessels resulted in remarkable necrosis of the tumor; ②Sinobufagin caused the vasculitis of the embolized vessels; and ③The embolic agents and anticancer drugs refluxed into gallbladder artery, leading to necrosis of the gallbladder mucosa (Figure 5). To prevent the occurrence of the second liver abscess and cholecystitis, it was essential to administer sufficient antibiotics after TSCTV.

In conclusion, based on anatomy of the vessels, TSCTV combines with the advantages of the three embolic agents so that occlusion of the tumor vessels was more complete and rational. The embolization effect was strengthened by SBMs causing endovasculitis and second occlusion. The structure of the "three-ply-board" can form a high concentration region of anticancer drugs. Three anticancer drugs complement with each other in pharmocologic action, thus augmenting the anticancer effect and reducing systemic side effects. From these results, it is concluded that the TSCTV is a ideal therapeutic regimen for HCC patients.

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Expression of glutathione S-transferase placental mRNA in hepatic preneoplastic lesions in rats *

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Subject headings liver neoplasms; glutathione S-transferase; in situ hybridization; RNA, messenger; precancerous conditions

Abstract

AIM To detect glutathione S-transferase placental (GST-P) mRNA expression in hepatic preneoplastic lesions in rats.

METHODS Using Solit-Farber model, the GST-P mRNA expression was observed in hepatic preneoplastic lesions induced by diethylnitrosamine (DEN) in rats and normal and regenerated hepatic tissues in the control group by in situ hybridization.

RESULTS GST-P mRNA was mainly expressed in altered hepatic foci (AHF) and some of the oval cells in hepatic preneoplastic lesions and the extent of its expression was different among various foci or/and positive cells in the same focus whereas no expression was observed in normal and regenerated hepatic tissues.

CONCLUSION Cells in AHF and oval cells may be the preneoplastic cells in the experimental hepatocellular carcinoma at the molecular level and heterogeneity exists in GST-P transcription levels.

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INTRODUCTION

Glutathione S-transferases (GSTs, EC2,5,1,18) are a family of dimeric proteins that may play important roles in both the intracellular transport of hydrophobic molecules and the metabolism of toxic compounds. The cytosolic GSTs can be divided into at least four different families, i. e. α, μ, π and θ based on N-terminal sequences, substrate specificities and affinity for non-substrate ligands in human. GST-P (the rat enzyme equivalent to human GST-π) was first found in placenta, but later also in kidney, lung and testis[1]. Recently, it has been shown by Northern blot and Dot blot analysis that GST-P mRNA is present abundantly in preneoplastic lesions and hepatocellular carcinoma induced by genotoxic carcinogen[2]. However, in hepatic preneoplastic lesions, which cell type has GST-P mRNA expression has not been well elucidated. This study was to investigate the expression of GST-P mRNA in hepatic preneoplastic lesions in rats by in situ hybridization and reveal the significance and regulation mechanism of its expression.

MATERIALS AND METHODS

Animal models and samples processing

Seventy male Wistar rats, weighing 100 g - 150 g, were randomly divided into experimental group (A) and control groups (B,C). Group A (30 rats), according to Solt-Farbar model[3], was initially given a single intraperitoneal injection of DEN at 200 mg/kg(Sigma product) dissolved in 0.9% NaCl to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they were fed with 0.02% 2-acetylaminofluorene (AAF, Sigma product) for 2 weeks. All the rats were subjected to partial hepatectomy (PH) at the 3rd week. Group B (20 rats) were injected with 0.9% NaCl instead of DEN solution and then underwent PH at the 3rd week. Group C (20 rats) were injected with 0.9% NaCl only (Figure 1).

Rats of each group were killed at the 1st, 2nd, 3rd, 4th and 5th week. The livers were removed. A portion of lesion tissues were stored at -80 ℃. Frozen serial sections were made for in situ hybridization and histochemical demonstration. The remainders were fixed in 4% paraformaldehyde for routine staining with hematoxylin and eosin.

Morphological observation and γ-GT histochemical staining

Pathomorphological observation and histochemical assay were made according to the National[4] and Rutenberg method[5].

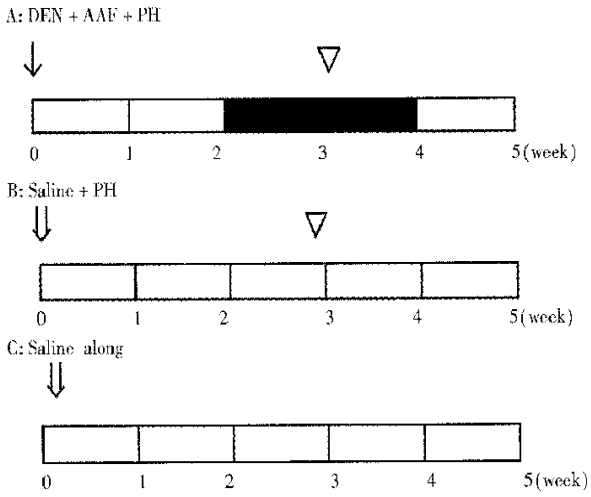

Figure 1 Expermental protocol
↓ DEN 200 ng/kg ip ⇩0.9% NaCl 0.5 ml/kg ip
▽PH:2/3 partial hepatectomy ■0.02% 2-AAF in diet

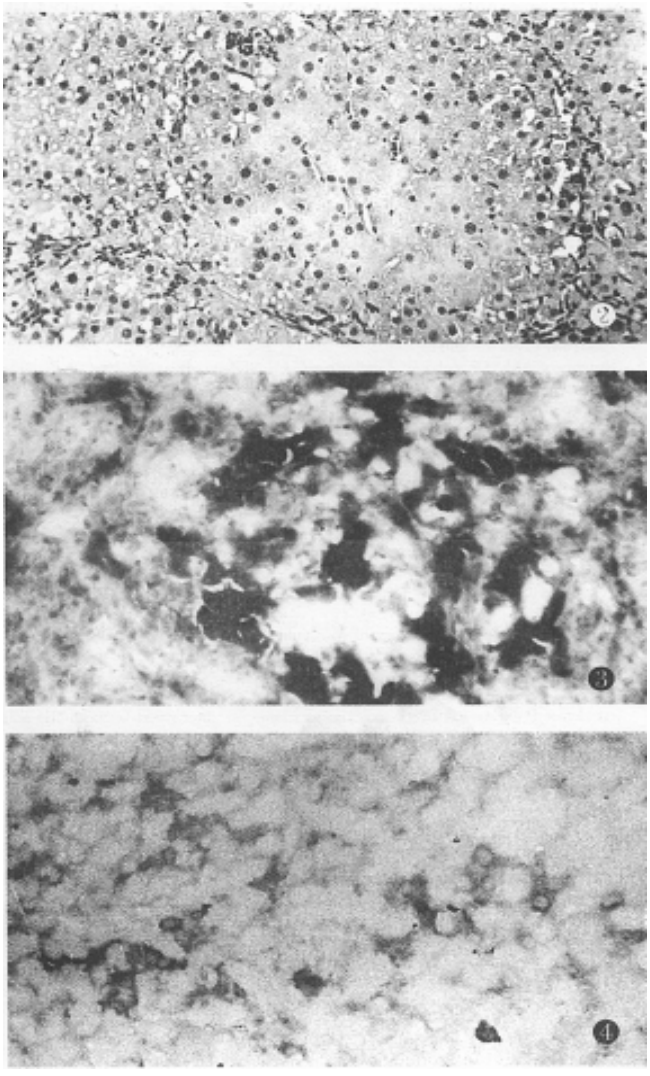

Figure 2 Altered hepatic- foci at the 5th week (HE×150)
Figure 3 γ-GT positive foci of liver cells at the 5th week (Enzymo histochemistry) ×200
Figure 4 GST-P mRNA positive foci of liver cells at the 5th week (ISH×400)

In situ hybridization

The bacteria carrying the pUC18 plasmid containing full length 0.75kb GST-P cDNA, PGP5 were gifts from Dr. Sugioka. The special restricted endunuclear enzymes cutting sites were EcoRI-SaLI. Probes were prepared according to J. Sambrook's method[6] and labeled by digoxigenin (DIG) using DIG-labelling and detection kit (Boehringer Mannheim Inc).

In situ hybridization was done according to SU HC *et al*[7]. Frozen sections were fixed in 4% paraformaldehyde/PBS and then rinsed with PBS, glycine/PBS and Triton-100/PBS in turn. Hybridization buffer contained 110 μg/L DIG-GST cDNA probes, 5 × SSC, 50% formamide, 5 × Denhardts solution, 5% dextron sulfate and predenatured 0.5 g/L ssDNA. Optimal hybridization temperature was about 42℃ in humid chamber. As a negative control, hybridization buffer did not contain DIG-GST-P cDNA probes, and sections were digested with RNase at 37℃ before hybridization.

RESULTS

Pathomorphological and histochemical changes

One week after PH, there was a grey granular appearance on gross inspection in experimental group rat liver. Histological examination of the liver tissues showed many AHF. These foci were mainly composed of basophilic cells. Proliferation of oval cells was found in portal areas (Figure 2). AHF was γ-GT positive (Figure 3).

In situ hybridization

At the 3rd week, GST-P mRNA was expressed in a few clusters of oval and liver cells in preneoplastic lesions. At the 4th and the 5th week, GST-P mRNA was mainly detected in hyperplastic AHF and some of the oval cells. Hybridization signal positive granules were mainly distributed in the cytoplasm of altered hepatic cells (Figure 4). The extent of the GST-P mRNA expression was different among the foci or/and positive cells in the same focus. Results of negative control showed only background staining, indicating the specificity of hybridization. No positive signals were found in control groups.

DISCUSSION

The binding of electrophilia metabolites of carcinogens to macromolecules, especially the DNA, is a critical event in chemical carcinogenesis. GST can detoxify a number of carcinogenic electrophiles, including dioleposide metabolites of polycyclic aromatic hydrocarbons by catalysis of the conjugation with reduced glutathione. Four multigene families of GSTs were characterized in human and rats, i. e., α, μ, π and θ[1]. The expression of xenobiotic metabolizing enzymes in

human hepatocellular carcinoma is complicated and a few findings support the proposal that GST-π could be used as a marker of hepatocellular carcinoma[8]. However, GST-P has been demonstrated to be a very useful marker enzyme, its mRNA is hardly detectable in normal rat livers, but is very strongly expressed in preneoplastic livers by induced genotoxic carcinogen by Northern blot and Dot blot hybridization methods[2]. There are many cell types in the liver tissues. Northern blot and Dot blot hybridization only showed an average level of the whole cell gene expression, not single cell gene expression and its characteristics. It provides the most accurate identification of RNA transcripts at single cell level by in situ hybridization technique. Our findings showed that GST-P mRNA was mainly expressed in AHF and some of the oval cells in hepatic preneoplastic lesions and the extent of its expession was different among foci or/and positive cells in the same focus whereas no expression was observed in normal and regenerated hepatic tissues. This study indicates that cells in AHF and oval cells may be the preneoplastic cells in experimental hepatocellular carcinoma at the molecular level and heterogeneity in GST-P transcript levels.

GST-P is known as a reliable tumor marker for chemical carcinogen-induced and spontaneously occurring preneoplastic lesions and hepatomas in rats. Thus, GST-P expression may be closely related to the process of hepatocarcinogenesis in rats. In multistep carcinogenesis, changes in the pattern of gene expression might be an important step in oncogene activation and/or inactivation of antioncogenes. To investigate possible alterations in gene expression pattern during neoplastic transformation, it may be useful to explore tumor marker gene expression. Why and how is the GST-P gene activated inevitably during the course of liver cell transformation Two possible mechanisms are considered: One is that the GST-P gene is located in close proximity to a gene that is directly involved in liver cell transformation and local chromosomal changes activate both genes simultaneously. The other is that those genes are not physically linked, but their expression is regulated by some common transcription factors. The GST-P gene has a strong enhancer element, GPE1, located at 2.5kb upstream from the cap site. The enhancer consists of the TRE-like sequence with palindromical orientation. GPE1 is a major control element responsible for GST-P expression in preneoplastic lesions. The regulatory region of the GST-P gene has another TRE-like sequence located at 61bp upstream from the cap site. These TRE-like sequences are active as AP-1 (Jun/Fos) binding sites and TREs, at least in vitro DNA binding and transfection analyses. From these results, it is inferred that the GST-P gene is controlled, at least in part by AP-1. However, AP-1 is not the only factor activating GPE1, because the expression of Jun and Fos do not always correlate with GST-P expression. Recently, the consense Maf recognition element (MARE) sequence was identified and is similar to the TRE sequence. Since the TRE sequence of the proximal region of the GST-P gene is internal to a MARE, Maf too might regulate GST-P expression. Sakai found that peroxisome proliferator activated receptor α (PPARα) can interact the Jun, not Maf, and inhibit the activation of GST-P expression[9-13].

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Cloning of 3H11 mAb variable region gene and expression of 3H11 human-mouse chimeric light chain *

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Subject headinsg gene, expression; stomach neoplasms; antibodies, monoclonal; chimeric antibody

Abstract

AIM To clone mouse anti-human gastric cancer mAb (3H11) variable genes and to construct 3H11 human-mouse chimericantibody.

METHODS The entire VH and VL genes of anti-gastric cancer mAb 3H11 were cloned by RT-PCR method from 3H11 hybridoma cells, using 5′ primers for leader sequences. The 3H11 VL gene was then inserted into human-mouse chimeric light chain expression vector and transfected into murine Sp2/0 myeloma cells.

RESULTS DNA sequence analysis indicated that the cloned genes included the whole leader sequences and the mature Ig variable region encoding sequences. Aftergene transfection, transient expression of chimeric light chain protein was detected.

CONCLUSION DNA sequences and transient expression indicated that the cloned gene was functional. This work laid basis for constructing 3H11 human-mouse chimeric antibody in the future.

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INTRODUCTION

The mouse mAb 3H11 was raised against human gastric cancer cells[1,2]. But 3H11 mAbs were derived from mouse hybridoma and their inherent immunogenicity in patients precluded its long-term use. In an attempt to circumvent this problem, chimeric antibodies in which the antigen-specific variable (V) regions of the mouse antibodies were joined to the constant (C) regions of human antibodies. These molecules should be much less immunogenic and hence be more suitable for application. In order to obtain the correct VL and VH genes, we designed 5' primers according to the leader sequences and cloned the entire VL and VH genes by RT-PCR method. The transient expression of chimeric light chain protein indicated that the cloned gene was functional in expression.

MATERIALS AND METHODS

Materials

Mouse 3H11 mAb hybridoma was provided by Biochemistry Department of Beijing Institute of Cancer Research. Chimeric light chain expression vector pAG4622 was a gift from S.L.Morrison (University of California, Los Angeles); γ-^{32}P-ATP was from Beijing Ya Hui LtD. Fmol DNA sequencing system, pGEM-T vector system, Tag enzyme and restriction enzymes were purchased from Promega. Lopofectin was from Gibco BRL.

Design of PCR primers

The 5' primers for amplification of mouse V genes were synthesized according to Coloma *et al*[3]. An extra VH5' primer was added according to newly published sequences data[4] (VHL4 in Table 1). The 5'primers were designed to hybridize to partially conserved sequences in the leader regions of VH and VL. The 3'primer for VL genes was located at V-C junction of VL. The 3'primer for VH genes was located at the boundary of VH and CH1, which was effective for IgG, IgG2a, IgG2b and IgG3 subclass. Restriction sites were incorporated into the primers as shown in Table 1(R = A/G S = C/G Y = C/T M = C/A K = T/G H = A/C/T D = G/T/A).

Cloning of VL and VH genes

Total RNA was isolated from 107 cells of 3H11 hydridoma cells using Trizol reagent from Gibco BRL. The VL and VH genes were amplified by RT-

PCR method, purified by agarose gel electrophoresis and cloned with pGEM-1 system.

DNA sequencing

The VL and VH genes were sequenced by dideoxy-mediated chain-termination method, using fmol DNA sequencing system (Promega).

Table 1 PCR primers for cloning variable genes of rearranged immunoglobulin light and heavy chains

5'Primers for VL Signal peptide
LL1: GGGGATATCCACCATGGAGACAGACACACTCCTGCTAT
LL2: GGGGATATCCACCATGGATTTTCAAGTGCAGATTTTCAG
LL3: GGGGATATCCACCATGGAGWCACAKWCTCAGGTCTTTRTA
LL4: GGGGATATCCACCATGKCCCCWRCTCAGYTYCTKGT
LL5: GGGGATATCCACCATGAAGTTGCCTGTTAGGCTGTTG
3'Primer for VK spanning CK and JK1,2&4
MVK: GGATACAGTTGGTGCAGTCGACTTACGTTTKATTTCCARCTT
5'Primers for VH signal peptide
VHL1: GGGGATATCCACCATGGRATGSAGCTGKGTMATSCTCTT
VHL2: GGGGATATCCACCATGRACTTCGGGYTGAGCTKGGTTTT
VHL3: GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT
VHL4: GGGGATATCCACCATGATRGTGTTRAGTCTTYTGTRCCTG
3'Primer for VH spanning CH1 and JH
MVH: GACHGATGGGGSTGTYGTGCTAGCTGNRGAGACDGTGA

Construction of chimeric light chain expression vector

The pGEM-T vector containing 3H11 VL gene was digested with EcoR V and SalI. The 3H11 VL gene was purifed by electrophoresis and electroelution and cloned into expression vector pAG4622. Transfection of DNA into Sp2/0 cells was accomplished by two methods: Lipofectin procedure: 20μg plasmid DNA was mixed with 40μl lipofectin and left at room temperature for 15 minutes. The mixture was added into 1.6×10^7 Sp2/0 cells in 2ml serum-free culture medium drop by drop. The cells were then cultured at 37℃ in a CO_2 incubator for 20 hours and 2ml RPMI1640 containing 20% fetal bovin serum was added. After further 72 hours incubation, the supernatant was collected.

Electroporation[3]: 20μg PvuII linearized plasmid DNA was added into 1.6×107 Sp2/0 cells. After 10 minutes in ice bath, the cells were electroporated at 960μF, 2kV/cm by GENE PULSER (Bio-RAD). After another 10 minutes in ice bath, the cells were transferred into culture flask that contained 10 ml RPMI1640 containing 20% fetal bovin serum. The cells were cultured at 37℃ in a CO_2 incubator for 72 hours and the supernatant was collected.

Detection of human-mouse chimeric light chain

The expression of chimeric light chain was determined by ELISA. Goat anti-human IgG Fab (Sigma) at 2.3 mg/L in 0.05M borate buffer, was absorbed overnight in a 96-well plate. Either a control or supernatant samples was added and incubated for 1h. This was followed by a secondary antibody coupled to horseradish peroxidase (HRP) (Sigma) and developed with OPD (0.5 g/L) (Sigma) and absorbance at 492nm. Between each addition, the plate was washed with phosphate-buffered saline with 0.05% Tween. The positive control was normal human blood serum, and the negative control was the supernatant of Sp2/0.

RESULTS

Cloning of 3H11 VL and VH genes

Total RNA was isolated from 3H11 cells and covered to cDNA. PCR amplification of VL and VH genes were done separately with sets of primer pairs. Agarose gelelectrophoresis showed that 3H11 VL gene was amplified with LL2 and VH gene with VHL1 (Figure 1)

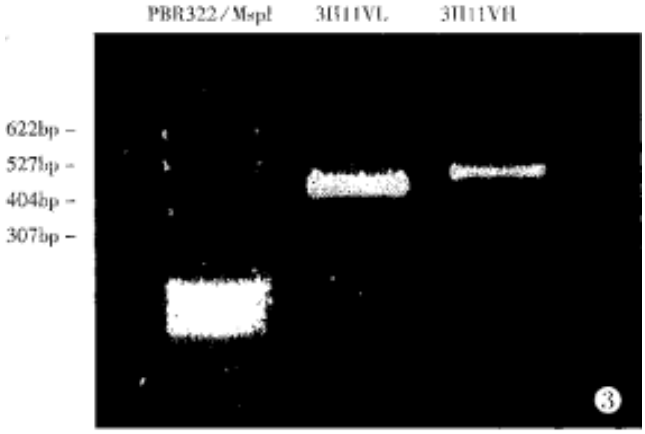

Figure 1 Amplification of variable genes of immunoglobulin light and heavy chains with PCR. The PCR products were resolved on 1.2% agarose gel.

Sequencing of 3H11 VL and VH genes

The purified VL and VH PCR products were cloned into pGEM-T vector and sequenced with fmol DNA sequencing system. Comparison of the sequences with published antibody variable region data[4] indicated that the 3H11 VL gene contained a leader sequence of 57 nucleotides encoding leader peptide of 19 amino acid residues, while the 3H11 VH gene had a 66 bases long leader sequences that encoded a 22 residues leader peptide (Figure 2). The sequences of the mature VL and VH regions were the same as what we cloned previously using primers for framework region[5].

Construction of chimeric light chain expression vector

The 3H11 VL fragments in pGEM-T were digested with EcoR V and SalI and ligated into pAG4622 vector (Figure 3). The resulting expression vector pAG4622-3H11 was verified by restriction enzyme analysis.

A.

```
-66-                                                          ATG GAT
                                                                M   D
-60-TTT CAA GTG CAG ATT TTC AGC TTG CTG CTA ATC AGT GTC ACA GTC ATA GTG TCT AAT GGA
     F   Q   V   Q   I   F   S   L   L   L   I   S   V   T   V   I   V   S   N   G
  1-CAA ATT GTA CTC ACC CAG  TCT CCA ACC ACC ATG GCT GCA TCT CCC GGG GAG AAG ATC ACT
     Q   I   V   L   T   Q   S   P   TCDR1 T   M   A   A   S   P   G   E   K   I   T
 61-ATC ACC TGC AGT GCC AGC TCA AGT ATA ACT TCC AAT TAC TTG CAT TGG TAT CAA CAG AGG
     I   T   C   S   A   S   S   S   I   T   S   N   YCDR2 L   H   W   Y   Q   Q   R
121-CCA GGA TTC TCC CCT AAA  CTC TTG ATT TAT AGG ACA TCC AAT CTG GCT TCT GGA GTC CCA
     P   G   F   S   P   K   L   L   I   Y   R   T   S   N   L   A   S   G   V   P
181-GTT  CGC TTC  AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATT GGC ACC ATG GAG
     V   R   F   S   G   S   G   S   G   T   S   Y   S   LCDR3 T   I   G   T   M   E
241-GCT GAA  GAT GTT GCC ACT TAC TAC TGC CAG CAG GGT AAT TAT TTA TCA CGC ACG TTC GGA
     A   E   D   V   A   T   Y   Y   C   Q   Q   G   N   Y   L   S   R   T   F   G
301-GGG GGG ACC AAG CTG GAA ATA AAA
     G   G   T   K   L   E   I   K
```

B.

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-57-       ATG GAA TGG AGC TGT GTC ATG CCC TTC ATC CTC TCA GGA ACT GCA GGT GTC CAC TCC
           M   E   W   S   C   V   M   P   F   I   L   S   G   T   A   T   V   H   S
  1-CAG GTT CAG CTG TGG CAG TCT GGA GCT GAG CTG GCG AGG CCC GGG GCT TCA GTG AAG CTG
     Q   V   Q   L   W   Q   S   G   A   E   L   A   R   P   G   A   S   V   K   L
                                                  CDR1
 61-TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC TAC TAT ATA AAC TGG GTG AAA CAG AGG
     S   C   K   A   S   G   Y   T   F   T   D   Y   Y   I   N   W   V   K   Q   R
                                                                  CDR2
121-ACT GGA CAG GGC CTT GAG TGG ATT GGA GAG ATT TAT CCT GGA AGT GGT AAT ACT TAC TAC
     T   G   Q   G   L   E   W   I   G   E   I   Y   P   G   S   G   N   T   Y   Y
181-1AAT GAG AAT TTT AAG GGC AAG GCT ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC
     N   E   N   F   K   G   K   A   T   L   T   A   D   K   S   S   S   T   A   Y
241-ATG CAG CTC ATT AGC CTG ACA TCT GAG GAC TCT GCA GTC TGT TTC TGT GCA AGA TAC TCT
     M   Q   L   I   S   L   T   S   E   D   S   A   V   C   F   C   A   R   Y   S
301-GGT TAC GAC GGA TAT TAC TAT GCT ATG GAC TAT TGG GGT CAA  GGA ACC TCA GTC  ACC GTC
     G   Y   D   G   Y   Y   Y   A   M   D   Y   W   G   Q   G   T   S   V   T   V
361-TCC TCA
     S   S
```

Figure 2 The nucleotide and amino acid sequences of 3H11 mAb VL(a) and VH(b) genes. Underlined sequences are CDR regions. Numbering according to mature protein sequences.

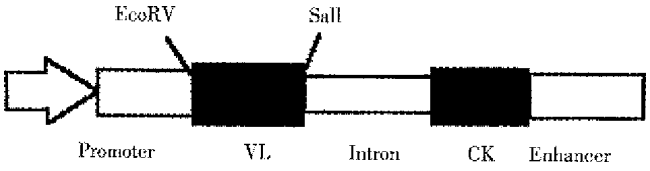

Figure 3 Human-mouse chimeric light chain expression vector pAG4622.

Table 2 Expression of chimeric light chain in transfectants determined by ELISA

ELISA	Supernatant			Human	Mouse
system	3H11	Sp2/0	Transfectant	Ig	Ig
Anti-human	0.169	0.112	1.214	1.660	0.122
Anti-mouse	1.564	0.147	0.128	0.102	1.538

a. Anti-human Fab and HRP-anti-human Fab Ab were used as catcher and second Ab.
b. Anti-mouse Fab and HRP-anti-mouse Fab Ab were used as catcher and second Ab.
c. ABS492

Expression of chimeric 3H11 light chain

The expression vector pAG4622-3H11 was transfected into murine myeloma Sp2/0 cells by lipofectin or electroporation. Culture supernatant was harvested after the transfected cells were incubated at 37℃ for 72 hours. Expression of chimeric 3H11 K chain was tested by ELISA. As shown in Table 2, human Ck was expressed in the culture supernatant of transfected cells indicated that the cloned gene was functional.

DISCUSSION

Monocloned antibodies (mAbs) are well-characterized highly specific reagents, and widely applied *in vitro* into immunochemical characterization and quantitation of antigens. They are being used clinically for both diagnosis and therapy increasingly. Their *in vivo* application is limited because most available mAbs are derived from mouse hybridomas, and their inherent immunogenicity in patients precludes their long-term administration. In an attempt to circumvent this problem, chimeric antibodies have been produced. Immunoglobulin V gene used to be

obtained by genomic library which was tedious and time-consuming. Recent developments in PCR technology have greatly facilitated the cloning of variable region genes[6]. Two approaches have been used to design the 5' primers. In one approach, degenerated primers for the 5'end are designed to recognize relatively conserved sequences within the 5' end of framework 1. Because the primers are within the coding sequences of the mature antibody and the introduction of restriction sites, amino acid substitutions that change the Ag-binding specificity or the affinity of the cloned antibody may result[7]. An alternative approach takes advantage of the conservation of the leader sequences. Since the leader is not present in the mature antibody molecules, any sequence alternations introduced by the degenerated primers will not influence antibody function. The phenomenon occurred in our work, the deduced N-terminal sequence of mature 3H11 VH protein had 4 amino acid residue discrepancies between "leader primer" amplified VH gene in this work (QUQLWQS) and previous "framework 1 primer" amplified VH gene (LLELVQS). While in 3H11 VL gene there were 2 discrepant residues, QIVLT of authentic VL versus DIVMT from "framework 1 primer" amplified VL[8]. Indeed the changes had profound influence on the binding of engineered 3H11 Ab molecules to gastric cancer cells (manuscript in preparation). In order to maintain the original sequences of the C terminal of the V regions, Coloma *et al*[3] suggested that the V genes be first amplified with 3'primer located at constant regions. Then a second set of 3' primers were designed according to the authentic sequences, and the V genes were PCR amplified again. Since there were only 4J segments for murine VL and VH genes respectively, we designed 3'primers for VL and VH genes that straddled the V-C junction and might at most cause one residue substitution in VL ($Leu^{106}\rightarrow Ile^{106}$ in JK5) or VH ($Ser^{113}\rightarrow Ala^{113}$) while the labour of cloning was greatly reduced.

Due to the diversity of Ab leader sequences, it would be more difficult to amplify V genes from leader by PCR[3]. There were some reports about RT-PCR method by primers for leader sequences abroad, but there is no report yet in our country. The facts that we had successfully amplified the variable genes not only of 3H11 but also of CD3 (manuscript in preparation) indicated that the primers were effective.

The amplified 3H11 VL gene was cloned into chimeric light chain expression vector and transfected into SP2/0 cells. Transient expression analysis demonstrated that the VL gene was functional, thus laid the basis for constructing 3H11 human-mouse chimeric antibody.

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Relationship between different sources of drinking water, water quality improvement and gastric cancer mortality in Changle County * -A retrospective-cohort study in high incidence area

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Subject headings stomach neoplasms/ mortality; water supply; risk factors; cohort studies; incidence; retrospective studies

Abstract

AIM To investigate the relationship between different sources of drinking water supply, water quality improvement and gastric cancer mortality rate in a high risk area.

METHODS A retrospective-cohort survey was carried out in all towns of this county to study the effect of different sources of drinking water supply and water quality improvement on gastric cancer mortality rate.

RESULTS The gastric cancer mortality rate among the population 124.05/105 drinking river water was obviously higher than that of drinking shallow well water (74.85/105) ($P < 0.01$) according to the Zhanggang Town 16 years accumulated data. The same pattern was presented in 7 towns after balancing the confounders. The gastric cancer mortality rate of population drinking river water was 86.03/105, which was higher than those drinking shallow well water (62.03/105) and tap water (29.78/105) ($P<0.01$). When the drinking water switched from river and well water to tap water, the gastric cancer incidence decreased to 30.33/105 and 26.10/105, and the gastric cancer mortality decreased by 59% and 57% respectively.

CONCLUSION The quality of drinking water is one of the important factors of increased incidence of gastric cancer in Changle County, and water quality improvement has a beneficial effect, but the cause of high gastric cancer incidence may be multi-factorial in this area.

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INTRODUCTION

Changle County is located in the south-east beach area of Fujian Province with a population of over 600 thousand and has 17 towns. The standardized gastric cancer mortality rate ranged from $60.18/10^5$ - $98.68/10^5$ between 1971-1990. The highest standardized mortality rate in male was $161.20/10^5$, remaining at a high level for a long time[1]. It is one of the highest gastric cancer incidence area in China. The result of a retrospective cohort study of six towns in this county had been reported[2], which indicated that the gastric cancer mortality rate is related to drinking water supply. The drinking water was surveyed between 1991 - 1992 throughout the county. The stratifying analysis results of the seven towns investigated are presented as follows.

MATERIALS AND METHODS

Reliability of data

A resident retrospective survey of deaths from all causes has been conducted four times since 1973. Diagnosis of gastric cancer death made by hospitals at county level or above was 77.99%, 81.64%, 71.46%, and 81.79% respectively. Diagnosis of grades I and II were 75.60%, 78.58%, 88.58% and 97.48% (grade I: postmortem; grade II: pathology). The reliability and integrity of the survey and data all met the quality requirements set by the National Cancer Prevention and Treatment Bureau "Death Cause Survey Handbook" and the Ministry of Public Health "Resident Death Cause Survey".

Type of drinking water sources

Type of drinking water sources was divided into river water (partly including pond, stream), shallow well (partly including deep well and spring water) and tap water. For calculating the person-year observed, only the people who must use one type water source for over four years can get into the cohort. It had been defined before the survey was begun because the drinking water source may be changed during the resident's lifetime. The types of drinking water drunk by people with gastric cancer before death were checked at the same time.

Data analysis and calculation

SAS software was used to analyze the data of this survey. Chisquare test was done by Mentel-Haneszel method. Gathering analysis for equilibrium of food, smoking and drink factors was proceeded by Systat software.

RESULT

Water type and gastric cancer mortality rate

Gastric cancer mortality rate in Zhanggang Town was the highest in this county from 1973 - 1990. Sixteen years accumulated data were analyzed, which indicated that gastric cancer mortality rate of population drinking river water was significantly higher than that of the shallow well source ($P < 0.01$) (Table 1).

Table 1 Gastric cancer mortality of Zhanggang Town according to the type of water supply (1/10^5) (1973 - 1990)

Type of water source	Male	Female	Total
River Water	176.91	57.29	121.05
	(141/79 699)*	(40/69 822)	(181/149 521)
Shallow well water	112.70	31.58	74.85
	(320/267 979)	(74/234 356)	(276/502 335)
RR	1.57	1.81	1.62
χ^2	19.88	9.49	28.79
P	<0.01	<0.01	<0.01

*Number of gastric cancer death cases / person-year observed.

In view of the fact that there were different factors among the towns in this county, such as geographical environment and living habits. According to the recent information[3], after controlling confounders of diet (mouldy grain, fresh vegetable, fish-sauce intake amount), smoking, drinking, etc., the different drinking water supply and gastric cancer mortality rates for the seven towns are presented in Table 2. The results showed that gastric cancer mortality rate of population with river water supply is higher than that of the shallow well water and tap water supply (P<0.01).

Table 2 Types of the drinking water supply and gastric cancer mortality rates for seven towns, Zhanggang (1/10^5)

Types of drinking water supply	Male	Female	Total
River water (1)	122.83	46.10	86.03
	(107/87 113)	(37/80 263)	(144/167 376)
Shallow well (2)	85.02	36.06	62.03
	(650/764 555)	(244/676 618)	(894/1 441 173)
Tap water (3)	51.31	3.86	29.78
	(16/31181)	(1/25906)	(17/57087)
RR	2.39	11.94	2.89
χ^2 (1:3)	10.617	8.618	18.005
P	<0.01	<0.01	<0.01
RR	1.66	9.34	2.08
χ^2 (2:3)	3.673	6.524	8.872
P	>0.01	>0.05	>0.01
RR	1.44	1.28	1.39
χ^2 (1:2)	12.158	1.686	13.017
P	<0.01	<0.05	<0.01

Water quality improved and gastric cancer mortality rate

Using population with persistent river and shallow well water supply as control group, the effect of the improved water quality on gastric cancer mortality rate was studied. The results showed that after switching river water or shallow water to tap water, the gastric cancer mortality rate presented a decreasing trend, especially in population that the river water was changed as summarized in Table 3.

Table 3 Improved water and gastric cancer mortality rates for seven towns in Changle County (1/10^5)

	Male			Female			Total		
	Person-year observed	No. of deaths	Death rate	Person-year observed	No. of deaths	Death rate	Person-year observed	No. of deaths	Death rate
Persistent river water supply	37 484	43	114.72	36 856	12	32.56	74 340	55	73.98
River water changed to tap water	35 088	14	39.90	24 262	4	16.49	59 350	18	30.33
RR		0.35			0.51			0.41	
χ^2		12.93			1.44			11.52	
P		0.0003[b]			0.2295			0.0007[b]	
Persistent well water supply	411 547	355	86.26	362 687	118	35.23	774 234	473	61.10
Well water changed to tap water	10 449	3	28.71	8 711	2	22.96	19 160	5	26.10
RR		0.33			0.71			0.43	
χ^2		3.98			0.24			3.80	
P		0.0460[a]			1.0000			0.0512[a]	

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

DISCUSSION

The relationship between different sources of drinking water and gastric cancer has been reported by several authors and comparatively consistent results were observed[4-7]. In the population with raw river water supply, the gastric cancer incidence was significantly higher than those with underground water supply. In our study, a retrospective-cohort of sixteen years accumulated data was analyzed. The result indicated that the type of drinking water was related to gastric cancer mortality. Even though the survey was extended to seven towns and under the condition that the confounders related to gastric cancer were balanced, the result showed the same pattern. In view of these results, the drinking water was thought to be one of important factors in the increased incidence rate of gastric cancer in this area.

According to the result of this investigation, altering drinking water has presented a significantly beneficial effect in decreasing gastric cancer rates. When the river water was changed to tap water, the rate decreased by 59% and 57% in case the well water was changed to tap water. Mutagenicity of different drinking water source has been compared in an earlier study which showed that the raw water had more mutagenic effects than the water purified in this area[8,9]. This result was identical with the results of epidemiological survey in this study. It is suggested that improving the drinking water quality should be a preventive method for decreasing the gastric cancer incidence rate in this high risk area. This study was only referred to one aspect of this problem, that is ' drinking water and gastric cancer'. Even if that drinking water had been improved in this district, the gastric cancer mortality rate was still on the median level. So the etiology of gastric cancer may be attributed to multi-factors in this high incidence area[10-12].

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Expression of somatostatin mRNA in various differentiated types of gastric carcinoma *

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Subject headings stomach neoplasms; somatostatin; RNA, messenger; in situ hybridization; immunohistochemistry

Abstract

AIM To investigate the expression of somatostatin mRNA in various differentiated types of gastric carcinoma.

METHODS By using in situ hybridization and immunohistochemical techniques, the expression of somatostatin mRNA and somatostatin immunoreactivity in the normal gastric mucosa, the poorly, moderately and well-differentiated gastric carcinomas, and various clinical stages of carcinoma were observed.

RESULTS In comparison with the normal gastric mucosa, the significantly increased expression of somatostatin mRNA positive cells was displayed in gastric carcinoma ($t = 2.681$, $P < 0.01$). The positive signal cells were distributed in a scattered form or aggregated as a mass or a cord, and the positive cells were more significantly enhanced in poorly differentiated carcinomas than those in well and moderately differentiated carcinomas ($t = 2.962$, $P < 0.01$). The somatostatin mRNA hybridization signals in stages III and IV of gastric carcinoma were significantly higher than those in stages I and II. The results of somatostatin immunoreactivity were consistent with those of in situhybridization.

CONCLUSION The alteration of the expression of somatostatin mRNA was associated with the development of gastric carcinoma and may play an important role in the process of tumor differentiation.

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INTRODUCTION

The study of the effect of endocrine hormone on the tumor development and cellular localization of the hormone DNA, and mRNA at molecular level using in situ hybridization techniques has been considered as the target of the tumor endocrinal investigation. It has been known that somatostatin involves the cell division and differentiation. However, there has been no report on somatostatin mRNA expression and its effect on normal and gastric carcinoma tissues with in situ hybridization at molecular level. In this study, in situ hybridization with digoxigenin-labelled antisense RNA probe and immunohistochemical techniques were used to investigate the alteration of somatostatin gene transcription and the expression of the above-mentioned tissues in the normal and the various differentiated types of gastric carcinoma tissues.

MATERIALS AND METHODS

Materials

Fifteen normal gastric mucosa and 32 gastric carcinoma samples were obtained from the surgical operations in Henan Tumor Hospital. Before operation no patients had been treated with anti-tumor drugs. After surgery, the specimens were rinsed immediately in saline and fixed in freshly prepared 4% polyformaldehyde (PFA) for 1-4 hours at 4℃. Washed with 0.01mol/L PBS, the specimens were immersed in autoclaved 30% sucrose overnight at 4℃. The frozen sections and paraffin-embedded sections were prepared and used for in situ hybridization and immunohistochemistry respectively. The frozen sections were incubated for 12 - 16 hours at 43℃, and then preserved at -20℃.

Slide treatment

Washed with water, the slides were immersed in clean solution overnight. Washed with water and distilled water, the slides were roasted for 2h at 180℃. Coated with DEPC created gelatin, the slides were dried overnight at 37℃.

Histopathological diagnosis and classification

The gastric carcinoma specimens were classified into poorly, moderately and well differentiated types, and four clinical stages (stages I - IV) according to the standard of the clinical classification for gastric carcinoma set up by the National Gastric Carcinoma Association.

In situ hybridization

The frozen sections of normal and gastric carcinoma tissues were treated with 0.1 mol/L PBS for 5 - 10 minutes, 0.1 mol/L glycine/PBS for 5 minutes, 0.3% Triton X-100/PBS for 15 minutes. Digested with 1 mg/L proteinase K (England) in 0.1 mol/L Tris-HCl, pH 8.0, 50 mmol/L EDTA buffer for 20 minutes at 37℃, the specimens were postfixed with 4% PFA for 5 minutes. Washed with 0.1 mol/L PBS, the specimens were immersed in freshly prepared 0.25% acetic anhydride in 0.1 mol/L triethanolamine for 10 minutes to reduce the background. Washed with 2×SSC for 10 minutes, each slide was covered with 10 μl - 30 μl hybridization solution (prehybridization procedure was omitted) containing 5% formamide, and 10% dextransulfate (Fluka), Digoxigenin-labelled SOM antisense RNA probe, 10 mmol/L Tris-HCl (pH 8.0), 0.03 mol/L NaCl, 1 mmol/L EDTA, 250 mg/L salmon sperm DNA, 10 mmol/L DTT, 0.25% PVP, 0.35% BSA, 0.24% Ficoll 400. The sections were covered with 20 mm × 20 mm Parafilm (Greenwich CT), and hybridization was performed at 43℃ for 16-20 hours in a moisture chamber. After hybridization, the specimens were washed with 2×SSC for 30 minutes. Following the removal of parafilm, the specimens were digested with 20mg/L RNase A for 30 minutes at 37℃ to eliminate the unhybridized probe. Washed 3 times with 0.05mol/L PBS for 5 minutes, the specimens were incubated with Anti-Dig-AP (Boehriger Mannheim) 1:1000 diluted in 1% BSA, 0.4% Triton X-100/0.05mol/L PBS for 12-16 hours at 4℃. Washed 4 times with 0.01mol/L PBS for 10 minutes, the specimens were stained with freshly prepared 400mg/L NBT/20mg/L BCIP for 3-16hours. The reaction was stopped by washing with 20mmol/L EDTA for 30 minutes.

Control experiment

The sections were pretreated with RNase A (0.05 g/L) for 30 minutes at 37℃. All procedures were the same as above except for no probe in hybridizationsolution.

Immunohistochemistry

Immunohistochemistry was used according to the PAP method. The samples were incubated with normal goat serum 1:10 diluted with 0.3% Triton X-100 for 30 minutes. Then, the somatostatin antibody (DAKO) (diluted 1 : 100 in PBS) was added and incubated for 24 hours at 37℃. Goat anti-rabbit IgG (diluted 1:25 in PBS) was added and incubated for 1 hour at 37℃. Then, PAP complex (diluted 1:100 in PBS) was added to the sections and incubated for 1 hour at 37℃. After that, the samples were colored in freshly prepared 0.5 g/L DAB for 10 - 15 minutes at room temperature.

Control experiment

Normal rabbit serum was substituted for goat anti-rabbit IgG and PBS was substituted for PAP complex.

Statistical treatment

The data were statistically analysed with *t* test.

RESULTS

Intracellular distribution of in situ hybridization positive signal

Distinct hybridization signal was detected with digoxigenin-labelled antisense RNA probe in both the normal tissues and in the gastric carcinoma tissues. The hybridization signal was blue-violet, and located in cytoplasm; and there was no signal in the nucleus. The background of the hybridization specimen was colorless.

Results of in situ hybridization in normal gastric mucosa

The positive signal cells were distributed in a scattered form. The hybridization signal could be seen in 13 of the 15 normal gastric mucosa specimens. However, the hybridization positive cells were less (Table 1), mainly located in the middle and the lower portion of the mucosa, and appeared in round or irregular shape (Figure 1), and some cells had a slender process.

Table 1 In situ hybridization results in normal and various differentiated types of gastric carcinoma (x±s)

Histological types	Cases	Positive cases	Positive cells / mm^{-2}
Normal gastric mucosa	15	13	$7.3±5.2^{b}$
Poorly differentiated carcinoma	9	9	$201.3±41.3^{d}$
Ring cell carcinoma	3	3	212.6±58.3
Moderately differentiated carcinoma	9	7	$40.0±18.4^{f}$
Well-differentiated carcinoma	11	9	$32.4±15.4^{b}$

$^{b}P<0.01$, $^{d}P<0.01$, $^{f}P<0.01$ *vs* carcinoma.

In situ hybridization results in the various differentiated gastric carcinoma

The expression of somatostatin mRNA in various differentiated gastric carcinomas was significantly higher than that in the normal tissues (Table 1). The positive cells were aggregated as a mass or a cord. The number of soma tostatin mRNA positive cells in the poorly differentiated carcinoma and ring cell carcinomas was increased more significantly than that in the moderately and well differentiated carcinoma tissues ($P<0.01$) (Figures 2-4).

Relationship between the in situ hybridization signals of somatostatin mRNA and the clinical stage of gastric carcinoma

The in situ hybridization signals detected with somatostatin antisense RNA probe in various clinical stages of gastric carcinoma tissues are displayed in Table 2.

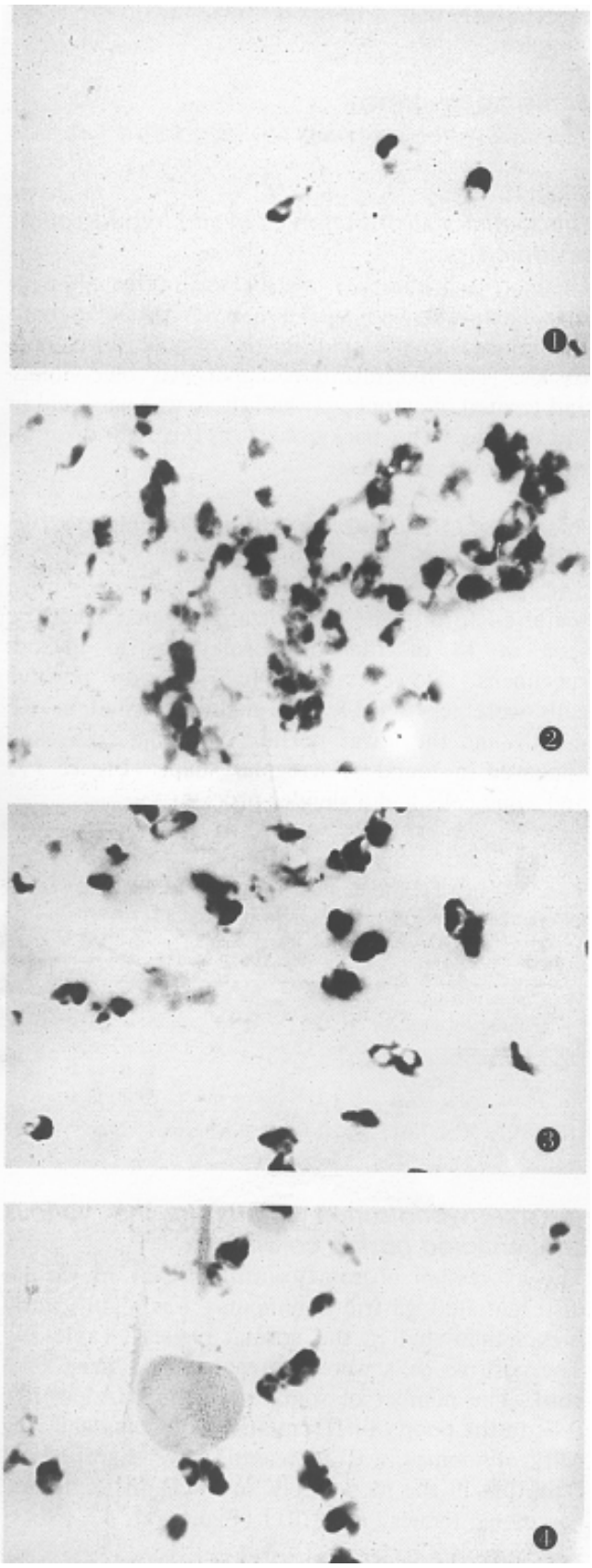

Figure 1 Somatostatin mRNA in normal gastric mucosa. ×400
Figure 2 Somatostatin mRNA in poorly differentiated gastric carcinoma. ×400
Figure 3 Somatostatin mRNA in moderately differentiated gastric carcinoma. ×400
Figure 4 Somatostatin mRNA in well differentiated gastric carcinoma. ×400

The results of somatostatin immunohistochemistry

The somatostatin positive immunoreactivity appeared as brownish colored granules and was located in the cytoplasm. The positive cells in the poorly differentiated carcinoma were significantly higher than those in the moderately and the well differentiated carcinomas. The results of somatostatin immunoreactivity was consistent with those by in situ hybridization (Table 3).

Table 2 Relationship between the in situ hybridization signals of somatostatin mRNA and clinical stages of gastric carcinoma

Clinical stage	Cases	Positive cases	Hybridization positive cells / mm^2
I	5	2	39.2±14.1
II	10	6	83.7±36.2[ab]
III	14	11	130.5±70.7
IV	3	1	133.5±64.3[d]

[a]P<0.05 *vs* I, [b]P<0.01 *vs* III; [d]P<0.01 *vs* II.

Table 3 The correlation analysis of the expression between somatostatin mRNA and somatostatin immunoreactivity

		Gastric carcinoma		
	Normal gastric mucosa	Poorly differentiated	Moderately differentiated	Well differentiated
r	0.874	0.725	0.836	0.836
P	<0.001	<0.001	<0.01	<0.01

Control experiment

Both in situ hybridization and immunohistochemistry were negative.

DISCUSSION

Up to date, there have been many reports about the relationship between gastroenteric hormones and endocrinal tumors in the digestive tract[1,2]. However, there have been few reports about the research of endocrinal regulation of gastroenteric non-endocrinal tumor and its mechanism with in situ hybridization. Chen[3] and Ooi[4] found endocrine granules or hormones in the common gastroenteric carcinoma cells and in the tumor endocrine cells of the digestive tract with ultrastructural and immunohistochemical techniques. However, immunohistochemical techniques could only show the gastroenteric hormone preserved in a certain kind of tumor cells, but could neither reveal whether the hormone came from protein synthesis by itself or was the exogenous substance, non distinguish if amino acid composition was similar, such as α or β cGRP[5] by proteins or polypeptides. In situ hybridization was not only the unique effective method to identify the result of immunohistochemistry, but also an index to watch

the hormone synthesis process, since the alteration of mRNA transcription rate was much faster than that of the translation production[6]. In this study, the in situ hybridization with digoxigenin-labelled somatostatin antisense RNA probe was first used to study the human normal and gastric carcinoma tissues. The results displayed intense signals with clear background and no nonspecific reaction, which indicated the satisfactory results of cellular localization at molecular level for somatostatin mRNA hybridization signal positive cells in normal gastric mucosa and the various differentiated gastric carcinoma tissues.

In the previous studies, it was believed that somatostatin inhibited cell growth[7]. However,the recent studiesdemonstrated that somatostatin could stimulate tumor cell growth[8,9]. Although the exact mechanism is still unclear, at least two possible sorts of explanation can be considered. The first is the inhibition of trophic action of gastrin on mucosal cells caused by exogenous and endogenous gastrin, and the second is the effect of somatostatin on cell proliferation. Our study on various clinical stages of gastric carcinoma showed that the somatostatin mRNA hybridization signals in stages III and IV were significantly higher than those in stages IV and II. Because there was a high incidence of migration to lymphonodes and peripheral organs in stage III and IV, it was indicated that the over expression of somatostatin was significantly correlated with the development and prognosis of gastric carcinoma.

Generally, the poorer the tumor cell differentiation, the worse the prognosis. In this study, the hybridization positive cells were more significantly enhanced in poorly differentiated gastric carcinoma than those in well and moderately differentiated gastric carcinomas ($P < 0.01$). The results indicated that, to some extent, somatostatin mRNA and somatostatin were associated with the differentiation induction of gastric carcinoma at the level of transcription and translation. The abnormal regulation at transcription level may stop the cell differentiation at a certain stage and the differentiated features of carcinoma cells occur. It is worth considering that the alteration of the expression of somatostatin mRNA may play an important role in the process of tumor differentiation.

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Arylsulfatase, β-galactosidase and lysozyme in gastric cancer cells and its relationship to invasion *

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Subject headings stomach neoplasms; hydrolases; proteoglycans histochemistry; neoplasm invasiveness; arylsulfatase; beta-galactosidase; muramidase

Abstract

AIM To study the distribution of arylsulfatase, β-galactosidase and lysozyme in gastric cancer cells, and its relationship to differentiation and invasion of gastric cancer cells.

METHODS Histochemical, immunohistochemical and ruthenium red (RR) electrocytochemical technique for three types of hydrolases and proteoglycans in pericancerous matrix in 33 cases of gastric cancer were observed under light and electron microscopy.

RESULTS The expression intensities of arylsulfatase, β-glactosidase and lysozyme in mucinous cell carcinomas were more intensive than those in well-differentiated and poorly-differentiated adenocarcinomas (P<0.05 - 0.01). The fibrous tissues smooth muscle and proteoglycans close to the cancer cells were degraded. They were found in the region far from the cancer cells. Expression of three enzymes mentioned above was low in adenocarcinoma cells, and fibrous tissues and RR granules were present and intact near the well-differentiated and poorly- differentiated adenocarcinoma cells.

CONCLUSION Mucinous cell carcinoma may release various hydrolases into extracellular matrix, inducing degradation of pericancerous matrix and facilitating cancer cell invasion and metastasis.

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INTRODUCTION

Tumor invasion and metastasis is a highly complex process. The mechanism of tumor invasion and metastasis is still not clear. In this paper the content and distribution of arylsulfatase β-galactosidase and lysozyme in three histological types of gastric cancer cells were studied by means of histochemical and immunohistochemical stains and the proteoglycans in pericancerous matrix by ruth enium red electrocytochemical stains respectively. The relationship between hydrolases and differentiation as well as invasion of gastric cancer cells were discussed.

MATERIALS AND METHODS

Surgically resected specimens of 33 cases of gastric cancer were studied. Histologically there were 14 cases of mucinous cell carcinoma, 9 cases of well-differentiated adenocarcinoma, and 10 cases of poorly-differentiated adenocarcinoma. Each specimen was treated as follows: ① The fresh tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5 μm section, and were stained by HE and lysozyme PAP immunohistochemical staining[1] (lysozyme antibody from Dako). ② Fresh tissue were fixed in 3% glutaraldehyde solution for 18 - 20 hours, and cut in a cryostat into 8 μm sections for arylsufatase and β-galactosidase histochemical staining[2,3]. Substrates were pnitrocatecholsulfate dipotassium salt and 5-bromo-4-chloro-3-indoxyl-β-galactoside (Sigma). ③ 0.1 mm^3 tissues from 20 of 33 cases of gastric cancer (including 10 cases of mucinous cellcarcinoma, 5 cases of well-differentiated and 5 cases of poorly-differentiated adenocarcinoma) were fixed in 2% ruthenium red (Merck) mixed with 4% glutaralde hyde in 0.1 M Na-cacoaylate buffer (pH 7.4). The proteoglycans of the extra-cellular matrix and the ultrastructures of cancer cells were observed under a Hitachi-600 electronmicroscope[4].

Grading of staining patterns

The staining patterns in tissue sections were scored according to the relative intensity and extent of positive staining. Weak positive (+): the number of positive cells less than 1/3 of the total cells, mode-rate reaction (++): 1/3 to 2/3 of the total, strong positive (+++): more than 2/3 of the total.

Statistics

The same enzyme staining grades from the tissues were compared by rank sum test.

RESULTS

Hematoxylin and eosin staining

In 14 cases of mucinous carcinoma, the cancer cells were diffusely distributed, some secreting a lot of mucin into extra-cellular matrix forming the mucin pool. There were no fibrous tissues and smooth muscle and lymphocyte infiltration in the pericancerous tissues. Nine cases of well-differentiated adenocarcinoma (including 2 cases of papillary carcinoma) displayed a tubular structure surroun ded by fibrous tissue proliferation or smooth muscle and lymphocyte infiltration. The cancer cells of 10 poorly-differentiated adenocarcinomas arranged in solid trabeculae of incomplete gland pattern accompanied by fibrous tissues and lymphocytes.

Histochemical staining of arylsulfatase

The positive reaction was dark-brown or yellow-brown in color. In cancer cells of mucinous carcinoma, the staining reaction was distributed in the cytoplasm and varied with the amount of cytoplasm, the more cytoplasm, the more staining intensity (Figure 1). There were light yellow-brown fine granules in the mucin pool. In well-differentiated adenocarcinomas the positive reaction was mostly located at the gland luminal border (Figure 2). In poorly-differentiated adenocarcinomas, most of cancer cells were negative, but were positive in the cytoplasm.

β-galactosidase histochemical staining

The enzyme-active sites were stained blue or turquoise. The reaction was most intense in mucinous cell carcinomas. The size of indigo particles was dependent on the cytoplasm, the particle was largest in abundant cytoplasm cells or signet-ring cells (Figure 3). Fine light blue granules were seen in the mucin-pool. In well-differentiated adenocarcinoma, the fine blue granules were mostly located in the apical cytoplasm of cancerous gland (Figure 4). In poorly-differentiated adenocarcinoma, some cancer cells were positive, and others were negative in reaction.

Lysozyme immunohistochemical staining

The positive reaction was yollow-brown. In mucinous cell carcinoma the positive product was diffusely distributed in the cytoplasm of most cancer cells, in signet-ring cell, the positive reaction was located on one side of the cytoplasm. In well-differentiated adenocarcinoma, positive product was located at the luminal side or in the cytoplasm in some cells, a portion of cancer cells were negative in reaction. In poorly-differentiated adenocarcinoma, most cancer cells were negative. The content and distribution of three type enzymes in gastric cancer cells are shown in Table 1.

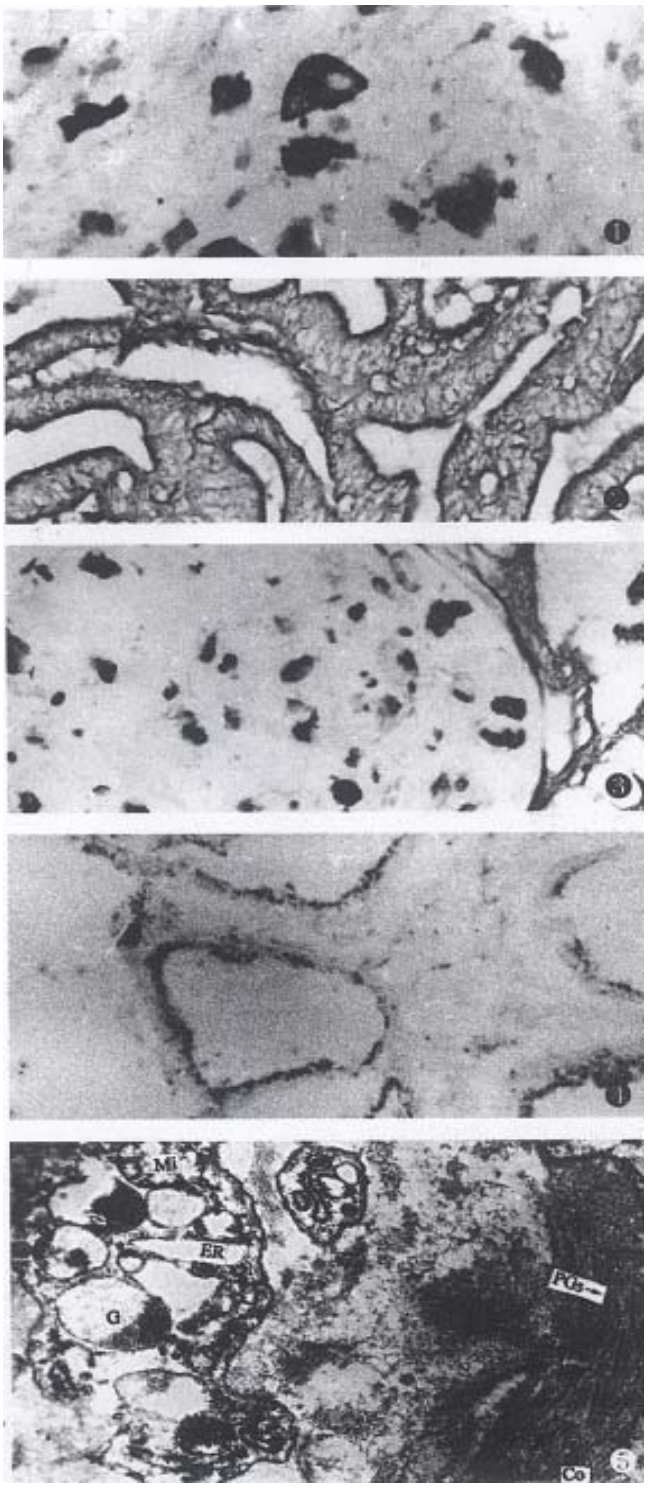

Figure 1 Mucinous cell carcinoma. The positive product of arylsulphatase is diffusely distributed in the cytoplasm. ×400

Figure 2 Well-differentiated adenocarcinoma. The positive product of arylsulphatase is located at gland luminal side. ×200

Figure 3 Mucinous cell carcinoma. The positive reaction of β-glactosidase is diffusely distributed in the cytoplasm. ×200

Figure 4 Well-differentiated adenocarcinoma. Fine blue granules are mostly located in the apical cytoplasm. The positive product is seen in macrophage at the matrix. ×200

Figure 5 Mucinous cell carcinoma. Mitochondria (Mi), endoplasmic reticulum (ER), secretory granules (G) in the cytoplasm were found. PGs (arrow) and collagens (Co) close to cancer cells are degraded. Both are seen in the region far from the cancer cells. RR×10 000

Talbe 1 Staining intensity of three hydrolases in gastric cancer

Histopathological types	No.	Arylsufatase[b]			β-galactosidase[d]			Lysozyme[c]		
		+	++	+++	+	++	+++	+	++	+++
Well-differentiated	9	7	2	0	5	2	2	8	1	0
Poorly-differentiated	10	8	2	0	7	3	0	7	3	0
Mucinous	14	3	4	7	2	5	7	4	3	7

[b]$P<0.01$, [d]$P<0.05$ [c]$P<0.05$.

These types of hydrolases in different types of gastric cancers were compared by rank sum test. There were significant differences between mucinous carcinoma and well-differentiated and poorly-differentiated adenocarcinoma ($P < 0.01$, $P<0.05$). There were no significant differences between well-differentiated and poorly-differentiated adenocarcinoma ($P>0.05$).

Ultrastructural findings

In mucinous carcinoma, the Golgi complex, endoplasmic reticulum, swollen mitochondria and secretory granules with various electron densities in the cytoplasm were easily found. RR granules and collagens close to the cancer cells of mucinous carcinoma were degraded but were observed in the region far from the cancer cells (Figure 5).

In well-differentiated adenocarcinoma, there were microvillus on the surface of luminal gland, and close connection and desmosomes between cancer cells. There were more swollen mitochondria, and smaller secretory granules with moderate density in the apical cytoplasm in some cancer cells. RR granules and collagens were present and intact near the adenocarcinoma. In poorly-differentiated adenocarcinoma, there were abundant ribosomes, a few secretory granules with moderate density in a few cancer cells. RR granules were found in surrounding cancer cells, which looked like short rods with 20 mm - 50 mm in diameter with high electron density, and attached on the collagens or located between collagens.

DISCUSSION

Relationship between hydrolases and differentiation

The results showed that Golgi complex, endoplasmic reticulum, secretory granules, and staining intensity of arylsulfatase, β-galactosidase and lysozyme were more intense in mucinous cell carcinoma and varied with cell differentiation. In well-differentiated adenocarcinoma, three kinds of hydrolases were mostly distributed in apical cytoplasm of luminal glands, electron-microscopically there were moderate electron density secretory granules in apical cytoplasm. In poorly-differentiated adenocarcinoma, there were few secretory granules, and, three types of enzymes were scanty. These results suggested that the content and distribution of these three enzymes are related to the differentiation and secretory function of cancer cells. The more differentiated of the secretory system, the more secretory granules in the cytoplasm and the more intensive enzymes staining reaction. Expression of three hydrolases in mucinous cell carcinoma was more intensive than those in well-differentiated and poorly-differentiated adenocarcinomas ($P < 0.05$). GERL (Golgi complex, endoplasmic reticulum and lysosome)[5] system was mature with a good secretory function, which may secrete various hydrolases.

Relationship between hydrolases and invasion

Malignant tumors may produce various hydrolases, which degrade pericancerous matrix at different sites in favour of the invasion[6].

Arylsulfatase and β-glactosidase were lysosomal enzymes which degrade proteoglycans (PGs) in the extracellular matrix and basement membrane. PGs are very complex macromolecules containing a protein core, to which one or more glycosaminoglycans (GAGs) chain are covalently bound and the chain radiate out from central core protein[7], owing to the polyanionic on the chains which tend to separate from each other to form the network structure, which can hinder the cancer cell spread. Arylsulfatase is a key enzyme in hydrolysing PGs. Removal of sulfate is a key step. Only after the sulfate group of PG was degraded, PG may be further hydrolyzed by other lysosomal enzymes. β-galactosidase is another important hydrolase which degrades the PGs. β-galactosidase not only cleaves the linkages between β-N-acetylglucosamine and β-galactoside (R-GlcNAc-Gal) on the GAG chains, but also degrade oxosylserine residues of the carbohydrate-protein linkage region on PGs[8], resulting in macromolecular PGs hydrolyzation, cancer cell spreading in matrix. Lysozyme is a representative enzyme of macrophage cells. The functional mechanism of lysozyme is to cleave the linkage between N-acetylmuramic acid and N-acetylglucosamine of mucopeptide in cell wall of bacteria[8], but, whether it may degrade similar structure on PGs and hyaluronide in extracellular matrix of human body to the bacteria has not been reported in literature. In the present study, three hydrolases were abundant in mucinous cell carcinoma, the positive reaction was also found in the extracellular mucous pool. PGs and collagens and smooth muscle in pericancerous matrix close to the cancer cell disappeared, which suggest that the cancer cells may secrete various hydrolases to degrade the extracellular matrix in different sites. So the cancer cells can easily invade and metastasize.

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A study of the relationship between trace element Mo and gastric cancer *

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Subject headings stamach neoplasms/ mortality; molybdenum/analysis; trace element; risk factors

Abstract

AIM To study the relationship between trace element Mo and gastric cancer.

MATERIALS AND METHODS Soil samples were collected according to its type in different areas of Jiangxi Province; available molybdenum content in soil was measured by catalytic polarography and rank correlation method was used to analyse correlation between the mean of soil available molybdenum and mortality rate of gastric cancer in each county and city in Jiangxi Province. Gastric cancer cases were selected from the authors' hospital, occiput hair was collected to measure its molybdenum content with an atomic absorption spectrograph and controls were selected from the same hospital for comparison. Gastric cancer cases were selected from three hospitals at the same time, blood samples were taken on an empty stomach and serum molybdenum contents were measured with the atomic absorption spectrograph, and controls were selected from the same hospitals. Blind method was used in the whole course (chemical analysts did not know the source and nature of samples).

RESULTS A negative correlation existed between soil available molybdenum content and mortality rate of gastric cancer (r = -0.285, P<0.05); hair molybdenum contents of gastric cancer cases were lower than those of healthy controls (0.308 μg/g ± 0.673 μg/g and 0.707 μg/g ± 0.561 μg/g respectively, P<0.01); serum molybdenum contents of patients were also lower than those of healthy controls (21.84 μg/L ± 7.49 μg/L and 25.38 μg/L ± 8.58 μg/L respectively, P<0.05).

CONCLUSION Deficiency of molybdenum may be one of the risk factors in gastric cancer.

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INTRODUCTION

A study of risk factors in gastric cancer in Jiangxi Province was started in 1983. The relationship between the soil trace element molybdenum (Mo) contents and mortality rates of gastric cancer in different areas of the province was investigated, and hair and serum Mo contents of gastric cancer cases and controls were determined.

MATERIALS AND METHODS

Eighty-five of 91 counties or cities in the whole Jiangxi Province were chosen for soil Mo determination. A total of 1748 soil samples were taken from farming areas. The characteristics of the representative areas were: nonirrigated farmland of 12 thousand mu; paddy field of 25 thousand mu, hilly and mountainous region (reclaimable wasteland) of 105 thousand mu. Samples were separately packed into plastic pockets, filtered through nylon sieves and digested by oxalic acid-ammonium oxalate. Mo in the extract thus obtained was measured by catalytic polarography. Twenty-three gastric cancer cases from the 2nd Affiliated Hospital of Jiangxi Medical College and 152 non-cancer controls of similar age and sex were chosen for the study. Occiput hair samples (3%) were obtained from each case and control, impregnated in detergent, rinsed, desiccated and acidified for the measurement of Mo contents with an atomic absorption spectrometer (HITACHI 8000).

Another group of gastric cancer and non-cancer matched controls were from the First and Second Affiliated Hospitals of Jiangxi Medical College and Jiangxi Tumour Hospital diagnosed from January to March in 1993 and 1994. Blood samples of these subjects were taken on empty stomachs in the morning. Serum Mo contents were determined with an atomic absorption spectrograph (SHIMATSU A- A 680, Japan).

RESULTS

Relationship between available molybdenum and mortality rate of gastric cancer

Soil samples were collected from different areas of the whole province, including gastric cancer high-incidence county Gui Xi and low-incidence county Hui Chang. About 20 samples were taken from each county or city. The mean of available Mo in soil was calculated. The correlation between the mean of soil available Mo and mortality rate of gastric cancer in each county or city was analysed by rank correlation. The result was r = -0.285, P < 0.01, which indicated a negative correlation between soil available Mo and mortality rate of gastric cancer.

Comparison of hair molybdenum between gastric cancer cases and healthy controls

Twenty-three gastric cancer cases were all definitely diagnosed by barium meal study, gastrocopy or biopsy. Among them, 18 were males and 5 females, aged from 30 to 70 years, averaging 52.2 ± 12.4. A total of 152 healthy persons served as controls. The mean of hair molybdenum content of the 23 gastric cancer cases was 0.308 μg/g ± 0.673 μg/g, being lower than that of the control group (0.707 μg/g ± 0.561 μg/g) ($P < 0.01$). The difference was significant.

Comparison of serum molybdenum between gastric cancer cases and non-cancer controls

There were 33 gastric cancer cases, with age ranging from 27 to 80 years (average 54.4 years), and a male: female ratio of 1.00 : 1.06. There were 79 non-cancer controls, aged from 29 to 83 (average 48.1 years) with a male:female ratio of 1.00:1.13.

Serum molybdenum contents were 21.84 ± 7.49 in the gastric cancer group and 25.38 ± 8.85 in the controls, ($t = 2.18$, $P<0.05$).

Serum molybdenum contents in gastric cancer cases were lower than those in non-cancer controls, the difference being significant.

DISCUSSION

Molybdenum has important physiological functions in human body. It is one of the essential trace elements. Low molybdenum content in soil has been thought to be one of the factors involving a high incidence of esophageal cancer and Keshan disease[1,2]. But there have been few reports about the relationship between trace element molybdenum and gastric cancer.

Our study showed a negative correlation ($r = -0.285$, $P < 0.01$) between soil Mo and mortality rate of gastric cancer. Soil available Mo contents were all low in these counties and cities with high mortality rate of gastric cancer such as Gui Xi. Lack of Mo in soil results in inadequate contents in corn and livestock which in turn, affects human body. Molybdenum is a component of nitrite reductase. In the process of the reduction of nitrate to nitrogen which can then be utilized by plants, the participation of molybdenum is indispensable. In the soil deficient of molybdenum, only part of nitrate can be reduced, thus leaving more nitrite in the environment and corn. This makes the human body and animals absorb and store more nitrites which under certain circumstances combine with nitrogenous substances to form nitrosamines, a group of strong carcinogens. Furthermore, molybdenum has some effects on the synthesis of vitamin C in plants, which may inhibit synthesis of nitroso-compounds in the human body[3]. To sum up, we can say that lack of molybdenum in the environment may obviously influence nitrogen fixation and transformation of soil nitrates as well as compound formation, and that Mo deficiency is definitely related to a high mortality rate of gastric cancer in local inhabitants.

Hair and serum molybdenum contents of gastric cancer cases were lower than those of healthy controls. The difference was statistically significant. This indicates that lack of molybdenum in the human body may be one of the causes of gastric cancer. Zhou *et al* found that molybdenum level in the mucosa of patients with gastritis or gastric ulcer was lower than that of normal people ($P< 0.01$), and that its level in mucosa of gastric cancer patients was also decreased[4]. This indicated that molybdenum deficiency may be an important factor in the causation of gastric disease. Wei *et al* used *N*-nitroso-sarcosine-ethyl (NSEE) induced mouse foregastric squamosum carcinoma model for studying the effect of molybdenum on carcinogenesis and proved that molybdenum had an obvious inhibitory effect on mouse foregastric squamosum carcinoma[5]. Miller[6] reported that most chemical carcinogens were metabolized by cytochrome P-450 in cells, producing “end-stage carcinogen” with electrophilic groups and then brought about cell deterioration. P-450 also had some relationship with its effect on the detoxification of carcinogens in the body. Lu *et al* studied the effect of molybdenum on the activities of P-450 and demethylase in rat liver and found that small dose of molybdenum could speed up detoxification of carcinogens and large dose of molybdenum might slow down that effect relatively[7]. Basing on the literature and our results, we can infer that some amount of molybdenum in the human body may lower the intake and thereby the contents of nitrate and nitrite in the body and enhance detoxification of carcinogens so as to give a protective action on the gastric mucosa. The content of molybdenum in human body may be related to molybdenum level in the environment. In arelative molybdenum-deficient environment, is human body also in a relative molybdenum-deficient status Is it useful to give a proper supplement of molybdenum to the people or give molybdenum-containing fertilizer for the prevention of carcinoma (especially against gastric cancer) in Modeficient areas These questions remain to be answered.

In conclusion, we have reasons to think that lack of molybdenum may be one of the risk factors in gastric cancer.

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Overexpression of P53 and its risk factors in esophageal cancer in urban areas of Xi'an

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Subject headings esophageal neoplasms; tumor suppressor gene; smoking; genes, p53; mutation; risk factors; immunohistochemistry

Abstract

AIM To investigate the risk factors of esophageal cancer (EC) in urban areas of Xi'an and to determine the association between overexpression of P53 and these risk factors.

METHODS All cases (89) and controls (97) were permanent residents in urban areas of Xi'an, all cases of primary EC had been histologically confirmed, controls were inpatients with non-cancer and nonsmoking-related disease. Cancer tissues and tissues adjacent to the cancer of 65 cases and 24 available normal esophageal tissues of controls were detected for P53 overexpression by the immunohistochemical method.

RESULTS The smoking and familial history of cancer were significantly associated with EC in Xi'an inhabitants. The laboratory assay indicated that P53 positive stain in EC was 50.0%(34/65)and 6.1%(4/65) in tissues adjacent to the cancer, but no positive stain was found in normal esophageal tissues of controls. The results showed that P53 overexpression in EC was closely related to smoking and cases with familial history of cancer.

CONCLUSION Smoking and familial cancer history were important risk factors for EC,and the alteration of P53 gene may be due to smoking and inheritance factors.

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INTRODUCTION

Esophageal cancer (EC) is the sixth most common cancer in males. In some areas of China, it is the leading cause of deaths. Previous studies suggested that certain local dietary and environmental factors are important risk factors in the high-incidence areas of China. But in Europe, epidemiological studies have shown that tobacco and alcohol consumption are the prevailing risk factors[1].

Recent progress in the molecular biology of cancer has a provided a basis for novel strategies in the prevention and treatment of cancer[2]. Cumulating evidence indicates that changes in both dominant oncogene and tumor suppressor genes are likely for malignant transformation of normal cells. Among these genetic abnormalities, the p53 tumor suppressor gene appears to be the most frequent target for DNA damage in carcinogenesis. Recent work suggests that proper function of this gene product in controlling growth is compromised by mutations through a significant region of the coding sequence[3], however, the exact cause of p53 gene mutations is still not clear until now. The current study was initiated to find out the risk factors of esophageal cancer in Xi' an inhabitants, and to correlate p53 overexpression with these factors.

Because almost all of previous studies in etiology of esophageal cancer were limited to study in the high-incidence areas of esophageal cancer, and in general, the high-incidence area tended to be rural[9], the results of these studies could not explain completely the etiology of esophageal cancer in Xi' an.

MATERIALS AND METHODS

Study design and inclusion criteria

The hospital-based case-control study was performed between August 1994 to January 1995. The subjects were selected from one of the three hospitals affiliated to the Fourth Military Medical University in Xi' an. All eligible subjects should be the permanent residents of the urban area of Xi' an. Informed consent was obtained from the subjects, if they agreed with the immunohistochemical and genetic analysis of tissue samples.

Our cases were selected from among consecutive persons who underwent major esophageal surgery and endoscopy, all were histologically confirmed as primary carcinoma of esophagus and were diagnosed for the first time, none of the case had chemotherapy or

radiotherapy prior to biopsies. Patients with esophageal cancer were excluded if they had a clinically apparent malignancy of any other origin.

Controls had a variety of diseases excluding cancer and diseases which are epidemiologically unrelated to tobacco smoking and aged from 45 to 65 years. Other malignancies were excluded by medical history and routine examinations.

Factors investigated

Factors in the questionnaire included demographic information (such as race, age, sex, educational level, and profession), tobacco smoking, alcohol consumption, intake of pickled vegetables, familial history of cancers, etc. All cases and controls were investigated by two trained investigators and interviewed for 20 minutes.

Tobacco smoking. Data on smoking history, i.e., duration of smoking, starting age, time of quitting, smoking, and average number of cigarettes consumed per day, were recorded.

Family history of cancer. Positive family history of cancer was defined as the presence of esophageal cancer in one or more relatives for both cases and controls. Data of three degree blood relatives on sex, age (age of death), cause of death, and duration of blood relatives living together with the subjects were investigated.

Samples

The tumor tissues were obtained from surgical or endoscopic resections. Nonpathologic or grossly normal esophageal tissues were obtained from the margins of the specimen or normal esophageal tissues apart from tumors under endoscopy. Tissue blocks of both grossly apparent carcinoma had nonlesional esophagus were fixed in 4% paraformaldehyde in standard phosphate-buffered saline (PBS), pH 7.4. They were irradiated with microwaves for 10-15 seconds and postfixed for 2 hours at 4℃ and then embedded in paraffin.

Immunohistochemistry

Thin 7 μm slices from paraffin-embedded specimens were deparaffinized routinely, then, added 50 μl 0.3% hydrogen peroxide for 10 minutes, followed by wash in PBS (pH 7.4) three times and treated with 50 μl 1% normal rabbit serum for 10 minutes at room temperature. The slices were incubated with primary antibody for 3 hours at room temperature in a moist chamber. The primary antibody used in this study was monoclonal antibody DO-7, purchased from Maxim Biotech Inc, China. The antibody reacted with wild and mutant type of the P53 protein. Immunostaining was performed by the streptavid in peroxidase conjugated method employing DAB as chromogens[4]. Specific staining was identified by the presence of brown or yellow reaction products.

Validity of laboratory measurement

All immunohistochemical studies were done without knowing the clinical data. To determine whether the positive stain resulted from contamination, two kinds of negative controls were used. Sections were incubated with PBS and non-immune mouse serum instead of the primary antibody. All sections for immunohistochemical analysis were observed by two experienced pathologists and their judgements should be in close agreement.

Statistical analysis

The odds ratio(OR) and its 95% confidence interval (CI) were calculated for variables that had been proved statistically significant in univariate analysis. Univariate analysis of factors potentially related to esophageal cancer and P53 overexpression were made to investigate the single effect of these factors. Logistic regression analysis was also performed. Univariate and multivariate analyses were conducted with SPLM program provided by the Department of Statistics in our university.

RESULTS

Subjects

Eighty-nine cases and 97 controls were collected. Their mean age (55.2 ± 9.7 years among cases and 54.4 ± 8.8 years among controls), proportion of gender, educational level and profession were similar. Detailed information on smoking and family cancer history was collected from all of our cases and controls.

The risk factors of esophageal cancer

The results indicated that the proportion of smokers and those with family histories of esophageal cancer was significantly higher than that in controls. It suggested that smoking and family cancer history were risk factors of EC. The OR for smokers was 3.26(95% CI 1.74 - 6.12), and for individuals with positive family cancer histories was 10.48(95% CI 4.81 - 22.48) (Table 1). Multiple logistic regression analysis showed the same results, in the logistic model, the interaction term was not statistically significant (probably due to sample size limitation) (Table 2).

Table 1 Single variable analysis of risk factors of EC

Factors	Cases	Controls	χ^2	OR	95% CI*	*P*
Smoking						
Yes	65	44				
No	24	53	15.53	3.26	1.74-6.12	0.0012
Family history of cancer						
Yes 43 8						
No	46	83	35.46	10.48	4.81-22.48	0.0000

*CI: confidence interval

Table 2 Logistic regression analysis of risk factors of EC

Factors	OR	Standard error	u value	*P*
Smoking	3.3	0.2939	4.0760	0.0000
Family history of cancer	3.0	0.2922	3.7090	0.0002

Table 3 Correlation between clinical features and overexpression of p53 in EC

Clinical features	P53 status		Univariate		Multivariate	
	Overexpression	Normal	χ^2	*P*	OR	*P*
Age	55.41±8.83*	55.32±11.07*	1.5836	0.9710	1.0104	0.8447
Sex						
Male	20	22				
Female	14	9	1.1497	0.5628	1.3611	0.7820
Smoking						
Yes	30	20				
No	4	11	3.8898	0.0486	3.1200	0.0041
Family history of cancer						
Yes	24	7				
No	10	24	17.2834	0.0001	15.8100	0.0002

*mean ± SD.

Immunohistochemical analysis

All cases, which were immunohistochemically detected, were squamous cell carcinomas. Among the 65 cases of esophageal cancer 52.0%(34/65) were positive for P53, 31 cases were negative for the immunostain. 6.1% (4/65) tissues adjacent to the carcinoma were positive for P53, but no positive products were observed in normal esophageal tissues of 24 controls. In the majority of tumor cells intense P53 immunostaining was observed in the cell nuclein and some tumor cells demonstrated the intense staining in cytoplasm. In normal esophageal mucosa, immunoreactivity of P53 was observed sporadically in basal and parabasal cells of mucosa. As a histological feature, the overexpression of p53 gene had heterogeneity in esophageal carcinoma. Immunoreactivity described earlier was not observed when incubated with PBS or non-immune mouse serum instead of the primary antibody.

Correlation of overexpression of P53 with clinical data

This study was unblinded after completing detection of overexpression of P53. Statistical analysis for the overexpression of P53 was then performed to correlate with clinical data. We could not find any statistically significant association between the overexpression of P53 and age, gender and occupation. In univariate analysis, the smoking and family histories of cancer were closely related with the overexpression of P53 ($P < 0.05$). Using the logistic regression model, multivariate analysis was performed to investigate the independent risk of smoking or family history of cancer as a predictive factor for overexpression of P53. Smoking and familial history of cancer were the factors shown to be closely related to overexpression of P53 after adjusting for other covariates ($P<0.01$), e.g., the odds ratio for overexpression of P53 to develop in smokers can be estimated at 3-fold increase over nonsmokers. The odds ratio for individuals who had family cancer histories was 15.8 (Table 3).

DISCUSSION

Numerous epidemiologic studies in China have shown that both particular environmental exposure and certain local dietary factors were responsible for the carcinogenesis of esophagus, but almost all previous studies were limited to the high-incidence areas of EC. Generally, the high-incidence areas have tended to be rural, so the results of these studies can not explain completely the rationale of EC in Xi'an which is not a high-incidence area in China. Our findings contradict the previous studies which stated that there was nosignificant association between smoking and esophageal cancer in China, and it was found that smoking and family history of cancer were important risk factors of EC.

More and more researches have shown the tumor is a molecular disease. Carcinogenesis depends on many genetic events, including mutation of oncogene or/and tumor suppressor gene. Carcinogens with significant mutagenic activity are components of various substances associated with tobacco smoking. P53 tumor supressor gene mutation has been observed in esophageal tumor cells induced by *N*-methyl-*N*-benzylnitrosamine treatment which is one of components in tobacco[5].

It is now clear that the p53 gene is the most frequent target gene among the known genetic alterations in EC[6]. P53 protein is a 53 KDa nuclear phosphoprotein and is expressed by many normal cells[7]. A decade ago, it was found that P53 protein was specifically overexpressed in transformed cells and undetectable in normal cells[8]. Numerous studies have confirmed these observations and shown that P53 protein accumulation is a consequence of its stabilization[9]. Normal P53 protein is known to be rapidly eliminated due to its short half-life, therefore, in normal cells, intracellular levels of P53 protein are low, and accumulation of P53 protein is usually the result of which modifies the conformation and stability of the protein[9,10]. It is easy to analyse the expression of P53 protein with immunohistochemistry on a large panel of tumors, because there appears to be a good correlation between the p53 gene mutation and its protein accumulation. In accordance with previous studies, P53 protein accumulation was found in 50% tumor specimens and 6.1% (4/65) tissues adjacent to cancer. In our study, in order to investigate whether the overexpression of P53 was present in normal esophageal tissues of controls, we immunostained 24 available specimens of controls, but no positive staining was found. The results suggest that the overexpression of p53 gene is a frequent genetic alteration and plays an important role in the carcinogenes is of esophageal carcinoma.

At present, the P53 tumor suppressor gene has come to the forefront of cancer research because it is commonly mutated in human cancer and the spectrum of p53 mutations in these cancers can provide clues to the etiology[11,12]. Various studies indicate that the p53 gene is a good target for molecular epide miological studies of various human cancer. Our epidemiological investigation also showed that major fraction of EC can be attributed to smoking and familial history of cancer, therefore, identifying the association of P53 overexpression with smoking and family history of cancer in this study is of particular interest. The study showed that the alteration of p53 gene was associated with smoking and family history of cancer.

Because p53 genes are inherited in a recessive form, requiring the loss of both copies for the phenotype to be expressed, it can be hypothesized that genetic predisposition to cancer induction may be related to inherited mutations in all ele of tumor suppressor genes that regulate cell growth and terminal differentiation, and this kind of inherited susceptibility to esophageal cancer can be provoked by the specific carcinogens contained in cigarettes. Inactivation or altered function of these genes results in increased risk for development of tumors. The results of this study may be a dramatic example of this process, suscep tible hosts who inherit one defective allele of p53 gene can become patients when another allele is lost through later somatic mutation induced by carcinogens in cigarettes.

As stated above, family cancer history depends on many factors, such as the number of relatives, their biologic relationship to the index case, their age distribution and the disease frequency in the population, so it can not be concluded that the positive familial history of cancer is due to genetic susceptibility, and several types of mutation do not lead to P53 accumulation and would be missed by immunohistochemistry[13], therefore, our further study will be focusing on a larger sample collection, PCR-SSCP analysis and DNA sequencing, and the application of more efficient statistical methods.

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Relationship between HBV viremia level of pregnant women and intrauterine infection: neated PCR for detection of HBV DNA

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Subject headings hepatitis B; DNA, viral/ analysis; radioimmunoassay; polymerase chain reaction; intrauterine infection

Abstract

AIM To determine the incidence of hepatitis B virus (HBV) in trauterine infection and to explore the relationship between HBV viremia level of pregnant women and HBV intrauterine infection.

METHODS Sixty-nine pregnant women were divided into three groups. Group A, 41 HBsAg positive patients, 14 of them were HBeAg positive (group A1), and 27 HBeAg negative (group A2); Group B, 12 HBsAg negative patients, but positive for anti-HBs and/or anti-HBe and/or anti-HBc; and Group C, 16 patients negative for all HBV markers. Blood samples of mothers were taken at delivery, samples of their infants were collected within 24 hours after birth (before injection of HBIG and HBV vaccine). All the serum samples were stored at - 20℃. HBV serum markers were tested by radioimmunoassay and HBV NDA were detected by nested polymerase chain reaction.

RESULTS In group C, all of 16 newborns were negative for HBsAg and HBV DNA. In group A, 7 infants were HBsAg positive (17.1%), and 17 (41.5%) were HBV DNA positive ($P < 0.05$). The incidence of intrauterine HBV infection was much higher in group A1 than that in group A2 (HBsAg 42.9% *vs* 3.7%, HBV DNA 92.9% *vs* 14.8%, $P < 0.05$). The incidence of HBV intrauterine infection was significantly different between high and low HBV viremia of mothers (93.3% *vs* 42.9%, $P<0.05$).

CONCLUSION The incidence of HBV intrauterine infection is high when HBV DNA in newborns detected with nested PCR is used as a marker of HBV infection. It is related to HBV viremia level of mothers.

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INTRODUCTION

The incidence of chronic HBV infection is high in China, more than 120 million people in China are carriers of HBV, 40% to 60% of them catch HBV infection from their mothers. So the key strategy for controlling HBV infection in China is to prevent HBV transmission from mother to infant. Transmission from mother to infant takes place in uterine, during delivery, and after birth. Vaccination after birth is of efficacy in preventing infant from HBV infection during delivery and after birth, but it can not interrupt HBV intrauterine infection. Previous studies showed that the HBV intrauterine infection rate was low (2.1% - 8.0%). However, recent investigations indicate that the rate is as high as 35% - 50%, indicating that intrauterine infection is the main route for HBV transmission from mother to infant[1-3]. We detected HBV DNA in the sera of newborns to determine HBV intrauterine infection rate and to explore its relation to HBV viremia level of mothers.

SUBJECTS AND METHODS

Subjects

Sixty-nine pregnant women and their newborns were investigated. All pregnant women were confirmed to be HBsAg positive by solid phase radioimmunoassay (spRIA), followed up and delivered at our hospital. They were divided into 3 groups. Group A, 41 cases positive for HBsAg, among them 14 were HBeAg positive (group A1) and 27 HBeAg negative (group A2). Group B, 12 cases negative for HBsAg but positive for anti-HBs and/or anti-HBe and/or anti-HBc (independent or combinant presence). Group C, 16 cases negative for all HBV markers.

None of the pregnant women had histories of hepatitis, symptoms and signs of hepatitis, threatened abortion, threatened premature delivery, and edema-hypertension-proteinuria syndrome. There was no significant difference in age, week of pregnancy at delivery, gravidity and parity among the three groups.

Methods

Blood samples of gravida were collected at delivery and the samples of newborns were taken within 24 hours after birth (before injection of hepatitis B vaccine and hepatitis B immunoglobulin). All serum

samples were stored at -20℃.

Serum HBV DNA was tested by nested polymerase chain reaction. Primers were designed according to the S region of HBV genome, synthesized by the Shanghai Institute of Cellular Biology. The sequences of primers are shown in Table 1.

Table 1 Sequences of primers

Number	Position	Sequence (5' - 3')
1	300-321	CATCTTCTTGTTGGTTCTTCTG
2	715-695	TTAGGGTTTAAATGTATACCC
3	421-441	TCTATGTTTCCCTCTTGTTGC
4	626-605	ACCACATCATCCATATATCTG

*1, 2 outer primers; 3, 4 inner primers

The product of first amplification was electrophoresed on 1.8% agarose gel, appearance of 416bp band was considered as strong positive for HBV DNA. If first amplification is negative, the product was used as plate for second amplification. The product of second PCR was electrophoresed on agarose gel, appearance of 206bp band was considered as weak positive for HBV DNA. Each sample was examined twice, there were positive, negative, and blank controls in each test.

Serum HBV markers were tested with solid phase radioimmunoassay (kits from 3V Company).

$X2$ test and direct calculation of probability on fourbolt data were used for statistical analysis.

RESULTS

Sixteen mothers in group C were negative for serum HBV DNA and their newborns were all negative for HBsAg and HBV DNA. The positive rates of HBsAg and HBV DNA in groups A and B are shown in Table 2.

In group A, the intrauterine infection rate was 17.1% (7/41) when HBsAg was used as a marker of intrauterine infection, the rate was high up to 41.5% (17/41) when HBV DNA was used, the difference was significant, $P < 0.05$. The HBV intrauterine infection was closely related to the mothers' HBeAg status (Table 2).

Table 2 Detection rate of HBsAg and HBV DNA

Group	Cases	Mother	Newborn	
		HBV DNA+ (%)	HBV DNA+ (%)	HBsAg+ (%)
A1	14	14 (100.0)	13 (92.9)	6 (42.9)
A2	27	7(25.9)	4 (14.8)	1 (3.7)
B	12	1 (8.3)	0	0

HBV intrauterine infection was related to mothers' status and levels of serum HBV DNA (Tables 3 and 4).

Table 3 Relationship between HBV intrauterine infection and mothers' HBV DNA status

Mothers' HBV DNA	No. of neonates	HBV DNA+	Intrauterine infection rate (%)
+	22	17	77.2
-	31	0	0

[b]$P<0.01$ *vs* mothers' HBV DNA positive

Table 4 Relationship between HBV intrauterine infection and mothers' serum HBV DNA levels

Mothers' HBV DNA level	No. of neonates	HBV DNA+	Intrauterine infection rate (%)
Strong positive	15	14	93.3
Weak positive	7	3	42.9

[a]$P<0.05$ *vs* mother HBV DNA weak positive.

DISCUSSION

Nested polymerase chain reaction (n-PCR) for HBV DNA detection is a sensitive and specific method for determining HBV intrauterine infection. The incidence of HBV intrauterine infection reported by different researchers is greatly discrepant, ranging from 2.1% to 50%, due to different sensitivities of methods used[3]. Yi *et al* detected HBV antigen in the liver of artificially aborted fetus with immunohistochemical assay and immuno-electromicroscopy, 43.75% of fetus from HBsAg positive pregnant women were infected with HBV[4]. Tang *et al* found that the HBV intrauterine infection rate was 44.4% (12/27) when HBV DAN in liver of fetus from HBsAg positive mothers was detected with Southern Blot, but the rate was only 18.5% when HBV antigen in fetal liver was detected by immunohistochemical method[5]. In this study, the HBV intrauterine infection rate was 17.1% when HBsAg in newborn serum detected by spRIA was used as a marker for diagnosis of intrauterine infection, but the rate was 41.5% when HBV DNA in newborn serum detected with n-PCR was used as a marker for diagnosis ($P < 0.05$). The results indicated that n-PCR for detecting HBV DNA in serum of newborn was a sensitive and specific method for diagnosis of HBV intrauterine infection. The sensitivity of spRIA for HBsAg was at ng level, and tissues and cells of fetus were not mature, resulting in low expression level of HBV antigen. So detection of HBsAg in newborn serum underestimated the incidence of HBV intrauterine infection[5]. The sensitivity of PCR for HBV DNA detection ranged from 10 fg/ml to l ag/ml. Nested PCR prevented the "plateau" of one-time amplification, and increased the sensitivity and specificity by changing the primers and plates[6]. Nested PCR for detection of serum HBV DNA in newborn had a more practical value than the methods used by Yi[4] and Tang[5], it can be used in clinical research into the mechanism of HBV intrauterine infection and in evaluation of the effect of methods for interrupting HBV intrauterine infection.

There is a closely positive correlation between the level of HBV viremia in mother and HBV intrauterine infection. HBeAg is a serum marker indicating HBV active replication. It has been reported that the HBV infection risk of infants from HBeAg positive mothers is 80% - 90%[1,7]. In our study all 14 HBeAg positive mothers had HBV DNA in serum, the HBV DNA detection rate of their newborns was as high as 92.9% (13/14). However, only 25.9% of HBeAg negative mothers were positive for HBV DNA, 14.8% (4/27) of their newborns were positive for HBV DNA in serum. These results show that the risk of HBV intrauterine infection is much higher in HBeAg positive mothers than that in negative ones ($P<0.05$).

The presence of HBV DNA is a direct marker of HBV active replication. The incidence of HBV intrauterine infection was much higher in newborns of mothers with HBV DNA than that of mothers without HBV DNA (77.3% *vs* 0, $P < 0.01$), and that the incidence was higher in newborns of mothers with high level of serum HBV DNA (strong positive for HBV DNA) than that of mothers with low level (weak positive for HBV DNA) of HBV DNA (93.3% *vs* 42.9%, $P<0.05$). These results confirmed that HBV intrauterine infection was positively related to the level of HBV replication in the mothers.

In conclusion, the incidence of HBV intrauterine infection is high, and it is positively related to the level of HBV replication in mothers. In order to control the epidemic of hepatitis B in China, it is important to explore the mechanism of HBV intrauterine infection and to develop effective methods for interrupting intrauterine infection. We have finished a prospective control trial, confirming that multiple injections of hepatitis B immunoglobulin during pregnancy can prevent fetus from HBV infection effectively (unpublished data).

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Immunohistochemical detection of HCV infection in patients with hepatocellular carcinoma and other liver diseases *

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Subject headings Hepatitis C; carcinoma, hepatocellular; immunohistochemistry; liver neoplasms; liver diseases

Abstract

AIM To detect HCV infection in patients with HCC and other liver diseases by the immunohistochemical method.

METHODS The expression of HCV antigen was identified by means of LSAB (labelled streptavidin-biotin) method using anti-NS3 monoclonal antibody.

RESULTS The positive rates of HCV antigen in the three groups of HCC, liver cirrhosis and hepatitis were 13.5% (7/52), 12.5% (2/16), and 10% (4/40) respectively, while in the samples from patients with constitutional jaundice and normal liver samples, no HCV antigen was found. HCV antigen could be seen in the nuclei and/or cytoplasms of carcinoma cells and/or pericancerous hepatocytes. In HCC, HCV antigen was more often seen in nuclei than in cytoplasms. The positive rate of HCV antigen in pericancerous tissues was higher than that in cancerous tissues.

CONCLUSION HCV is associated with HCC, and HCV infection enhances the development of liver diseases. HCV affects the initiative period of HCC and induces the malignant phenotypic alteration of hepatocytes.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common malignant tumors which ranks the third in cancer mortality in our country, while its etiology and carcinogenesis are still far from clearly identified. The association of hepatitis C virus (HCV) infection with HCC has been indicated by serosurvey[1,2], but studies at cellular level in detecting HCV antigen in liver tissues to demonstrate this association have so far been rare. The significance of results from a few immunohistochemical studies reported is quite limited because of the use of polyclonal antibodies and a small number of cases. In order to reveal the HCV infection status in HCC and other liver diseases and to explore the relationship between HCV and HCC at cellular level, we detected immunohistochemically the HCV antigen expression in cancerous tissues and liver tissues of 116 cases of different liver diseases (mainly HCC) using LSAB (labelled streptavidin-biotin) method and monoclonal antibody against NS3 antigen of HCV.

MATERIALS AND METHODS

Patients and samples

Liver tissue samples were obtained from inpatients during resection of their HCC in the Department of Abdominal Surgery of Affiliated Tumor Hospital of Sun Yat-Sen University of Medical Sciences in the period from April 1993 to Febrary 1994 and the preserved paraffin embedded samples by liver biopsy in the Department of Pathology of First Affiliated Hospital of Xinjiang Medical College from 1986 to 1990 were also collected for study. The clinical data are shown in Table 1. The diagnosis was made according to pathological examination, clinical data and laboratory assay.

Table 1 Clinical data of 116 cases of liver diseases

Groups	Cases	Male/famale	Age (years) ($\bar{x}$±s)	ALT (IU/L) ($\bar{x}$±s)
HCC	52	41/11	42±2	126±93
Cir.	16	10/6	42±6	171±68
CH	40	28/12	35±9	269±51
CJ	8	5/3	23±1	76±45

HCC: hepatocellular carcinoma; Cir: cirrhosis; CH: chronic hepatitis; CJ: constitutional jaundice

Reagents

The monoclonal antibody against NS3 antigen of HCV, prepared by Bionikes Co. U. S. A., was kindly provided by the Virology Institute of Chinese Academy of Preventive Medicine; LSAB (labelled streptavidin-biotin) kit and monoclonal antibody to HBsAg were obtained commercially (LSAB kit from DAKO, anti-HBs from Clinical Immunology Lab of Tongji Medical University).

Immunohistochemical methods

The tissue sections were dewaxed routinely and then treated as follows: 0.3% H_2O_2-methanol blocking for 20min, phosphate-buffered NaCl solution (PBS) washing, incubation with monoclonal antibody against NS3 of HCV overnight at 4℃, continuing the following procedure according to the instruction of LSAB kit, DAB staining.

The negative controls included: ①substitution of monoclonal antibody against NS3 of HCV with unrelated antibody (anti-HBs) or PBS, ②exclusion of incubation with antibody against NS3 of HCV, and ③normal liver samples as controls.

RESULTS

The positive rates of HCV antigen in the groups of different liver diseases

In the groups of HCC, cirrhosis and chronic hepatitis, the positive rates of HCV Ag were 13.5% (7/52), 12.5% (2/16) and 10% (4/40) respectively, while in the samples from constitutional jaundice and normal liver no HCV Ag was found. No positive staining was shown in the negative controls except with anti-HBs as the first antibody (the HBsAg staining was located differently, as compared with HCV antigen).

Location and distribution of HCV antigen in tissues

HCV Ag could be seen in the nuclei and/or cytoplasms of cancer cells and/or pericancerous hepatocytes of HCC. In the 7 positive cases of HCC, HCV Ag was more often seen in nucleus than in cytoplasm (5 cases in nuclei, only 2 cases in cytoplasms). This difference was not significant in the other groups. The HCV Ag positive cells were scattered singly or gathered in small groups in some parts. The detecting rate of HCV Ag in cancer tissues was 5.8% (3/52), whereas in the pericancerous tissues it was as high as 12.5% (6/48), significantly higher than that in the former.

DISCUSSION

Since the application of ELISA and PCR techniques in the detection of HCV antibody and HCV RNA, the view that HCV infection was an important risk factor for the genesis of HCC was supported by many studies which were mainly carried out in HBV non-endemic areas such as Japan and Europe[3,4]. Even in China, as an HBV hyperendemic country, the association of HCV infection with HCC was also preliminarily demonstrated, but the published investigations up to now were almost all serological stu-dies. Detection of HCV antigen at cellular level in liver tissues to demonstrate this association has been reported rarely. The specificity of the results from a few immunohistochemical studies reported was quite limited because of the use of polyclonal antibody derived from patient's serum, and the consistency of the results from different studies was quite poor[5]. We detected HCV antigen expression in cancerous tissues and pericancerous tissues of HCC using LSAB method and monoclonal antibody against NS3 antigen of HCV, and our results supported the association of HCV infection with HCC. The positive rate of HCV antigen in pericancerous hepatocytes was higher than that in cancer tissues, similar to the results of HCV RNA detection reported by Ohkoshi[6], and this phenomenon gives us two hints: HCV probably affects mainly the initiative period of HCC development and enhances malignant phenotypic alteration of normal hepatocytes; and as soon as the malignant alteration has taken place, the compatibility of hepatocytes to HCV decreases. The positive rates of HCV antigen in cases of HCC and cirrhosis were higher than those in cases of chronic hepatitis, constitutional jaundice and normal liver tissues and this result suggests that HCV infection may enhance the development of liver diseases. It is common knowledge that HCV, as a non-reverse transcription RNA virus, completes its proliferation cycle in cytoplasm, and so the expression of HCV antigen in nucleus is not related with the proliferation cycle itself. Whether HCV NS3 antigen, as a "counter-modulating" factor, gets into the nucleus to influence expression and regulation of host cell genes and to induce malignant alteration of cells remains to be studied further in the future.

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A new rat model of portal hypertension induced by intraportal injection of microspheres

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Subject headings portal vein; hypertension, portal; disease model, animal; latex microsphere

Abstract

AIM To produce a new rat model of portal hypertension by intraportal injection of microspheres.

METHODS Measured aliquots of single or different-sized microspheres (15,40,80μm) were injected into the portal vein to block intrahepatic portal radicals. The resultant changes in arterial,portal,hepatic venous and splenic pulp pressures were monitored. The liver and lungs were excised for histological examination.

RESULTS Portal venous pressure was elevated from basal value of 0.89 - 1.02 kPa to a steady-state of 1.98 - 3.19 kPa following the sequential injections of single- or different-sized microspheres, with a markedly lowered mean arterial pressure. However, a small-dose injection of 80μm microspheres (1.8×10^5) produced a steady-state portal venous pressure of 2.53×0.17 kPa,and all rats showed normal arterial pressures. In addition, numerous microspheres were found in the lungs in all experimental groups.

CONCLUSION Portal hypertension can be reproduced in rats by intraportal injection of microspheres at a small dose of 80 μm (1.8×10^5). Intrahepatic portal-systemic shunts probably exist in the normal rat liver.

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INTRODUCTION

Portal hypertension is associated with gross haemodynamic disturbances in portal and systemic circulations. Animal models are still important for research into portal hypertension. One of the most popular models used is the partial portal venous ligation model in the rat[1], but this can only achieve extrahepatic portal venous occlusion and is not representative of intrahepatic portal hypertension as seen clinically. Carbon tetrachloride induced models of cirrhosis closely resemble the major features of the human disease[2], but take a long time to develop and are associated with a high mortality and a wide heterogeneity in the stage and development of cirrhosis[3,4]. In the present study, a new rat model of portal hypertension was successfully induced by intraportal injection of microspheres.

MATERIALS AND METHODS

Animals

Thirty-eight Sprague-Dawley rats weighing 250 g - 350 g were randomly divided into six groups. Groups 1 (n = 6) and 2 (n = 6) received sequential injections of single-sized microspheres of 15 and 80 μm diameters respectively. Groups 3 (n = 6) and 4 (n = 6) were given sequential injections of different-sized microspheres in order of size 15, 40 and 80 μm and 80, 40 and 15 μm, respectively. According to the results of the four groups above, two bolus injections of 80 μm microspheres were selected as the suitable dose for the induction of portal hypertension in the model and these were given to Group 5 (n = 8) for further observation. Rats in Group 6 (n = 6) were injected with saline and served as controls.

Measurement of pressures

The animals were anaesthetised with fentany/ fluanisone (0.3 ml/kg, subcutaneously) and midazolam (0.3 ml/kg, subcutaneously). Mean arterial pressure (MAP) was monitored using a catheter in the left carotid artery. The abdomen was opened via a midline incision and the portal vein was cannulated through an ileocolic vein for measurement of portal venous pressure (PVP) and injection of microspheres. The splenic pulp pressure (SPP) was measured through a 23G butterfly scalp needle. Wedged hepatic venous pressure (WHVP) was measured in Groups 1,2,5 and 6. All of the cannulae were connected to P23XL (Viggo Spectramed Inc.) pressure transducers, and

permanent recordings were made on a polygraph recorder (Grass Instruments Inc., USA). When steady basal pressures had been achieved for at least 5 minutes, injections of microspheres or saline started. Before injection, latex microspheres (Coulter Electronics Ltd., England) were agitated for 60 seconds. In each injection, microspheres were given iav the portal venous catheter in a volume of 0.2 ml and immediately followed by 0.2 ml saline injection to flush the catheter. Only when a steady PVP has been achieved for at least 5 minutes was the next injection given. In Group 5 there was no interval between the two injections. The numbers of microspheres used are shown in Table 1. Following completion of the injection, animals were observed for 10 - 30 minutes until the final steady PVP had been reached. Finally a vascular clamp was applied to the portal vein at the liver hilus and the pressures monitored. After this, the animals in Group 6 received partial portal venous ligation. At the end of the experiment, all rats were killed by opening the chest. The liver and lungs were taken and fixed in 10% formal-saline for histological examination.

Table 1 Intraportally injected aliquots of microspheres

Group	Sphere diameter (μm)	No of aliquots	No of spheres / aliquot	Total No of spheres
1	15	6	5.6×10^6	3.4×10^7
2	80	5	9.0×10^4	4.5×10^5
3	15,40,80	15μm×2	5.6×10^6	1.1×10^7
		40μm×2	2.4×10^5	4.8×10^5
		80μm×2	9.0×10^4	1.8×10^5
4	80,40,15	80μm×2	9.0×10^4	1.8×10^5
		40μm×2	2.4×10^5	4.8×10^5
		15μm×2	5.6×10^6	1.1×10^7
5	80	2	9.0×10^4	1.8×10^5

Note: Pressures showed no changes after the 4th and 5th injections in Group 2. Therefore the 6th injection was not given.

Statistics

Results were expressed as mean ± standard error. Comparisons were made by means of *t* test. Results were considered statistically significant at $P<0.05$.

RESULTS

MAP decreased by approximately 45% following the first injection of microspheres in all experimental groups (kPa, 12.95 ± 1.66 *vs* 7.32 ± 0.68, $P < 0.001$). MAP in Group 5 eventually returned to normal levels after approximately 40 minutes following the two injections of 80 μm microspheres. No change in MAP was observed in the control rats during saline injections and after partial portal venous ligation. However, portal venous occlusion in control rats produced a significant reduction in MAP (kPa, 12.86 ± 1.65 *vs* 6.88 ± 1.14, $P < 0.001$), si-milar to that seen after microsphere injections in the experimental groups ($P > 0.05$).

PVP rose gradually following the microsphere injections and, in Group 1, synchronous increase in WHVP was found (kPa, 0.93 ± 0.13 *vs* 1.65 ± 0.24, $P < 0.01$) (Figure 1). In Group 2, 80 μm diameter microspheres produced a large, rapid increase in PVP (Figure 2) with a significant reduction in WHVP (kPa, 0.89 ± 0.09 *vs* 0.47 ± 0.09, $P < 0.01$). In Groups 3 and 4, a marked increase in PVP was only observed after injection of 80 μm diameter spheres (Figure 3). Four rats in Group 4 died within 2 - 17 minutes after the 6th injection of microspheres. In Group 5, two sequential bolus injections of 80 μm microspheres elicited animmediate reduction in WHVP (kPa, 0.92 ± 0.13 *vs* 0.47 ± 0.09, $P < 0.01$) and an immediate increase in PVP to $2.53 \text{ kPa} \pm 0.17 \text{ kPa}$, which remained elevated during the observation period of 150 minutes. There were no significant changes in PVP in the control group after injection of an equivalent volume of saline aliquots. Results are compared in Table 2.

Portal venous occlusion with a clamp produced a further large, rapid rise in PVP (Figure 1) to $5.16 \text{ kPa} \pm 0.76 \text{ kPa}$, $4.87 \text{ kPa} \pm 0.79 \text{ kPa}$, $4.95 \text{ kPa} \pm 0.99 \text{ kPa}$, $5.55 \text{ kPa} \pm 0.89 \text{ kPa}$ and $5.75 \text{ kPa} \pm 0.32 \text{ kPa}$ in groups 1,2,3,5 and the control respectively. There was no significant difference between the values in these groups ($P > 0.05$). Partial portal venous ligation in control rats induced a significant increase in PVP to $2.75 \text{ kPa} \pm 0.07 \text{ kPa}$ and was similar to that in Group 5 ($P > 0.05$).

Table 2 Comparison of changes in portal venous pressure (PVP) and splenic pulp pressure (SPP) in different groups after microsphere injections

Group	PVP(kPa)				SPP(kPa)			
	Basal	Post-infusion	Basal-Post	% increase	Basal	Post-infusion	Basal-Post	% increase
1	0.98±0.21	1.98±0.48[a]	1.00±0.37[a]	102.2±35.6[a]	1.17±0.39	2.01±0.51[a]	0.84±0.44[a]	90.9±41.8
2	0.89±0.19	3.19±0.29[a]	2.30±0.36[a]	272.2±78.0[a]	1.44±0.43	3.03±0.33[a]	1.60±0.60[a]	139.3±51.7
3	1.02±0.11	2.65±0.49	1.64±0.52	162.8±60.1	1.24±0.16	2.49±0.45	1.24±0.48	103.1±48.5
4	0.98±0.13	2.70±0.13	1.72±0.20	178.6±41.0	1.16±0.19	2.63±0.13	1.49±0.23	133.5±39.2
5	1.04±0.12	2.53±0.17	1.45±0.23[c]	140.3±32.0[c]	1.44±0.19	2.47±0.19	1.04±0.28[c]	74.9±26.6[a]
6(control)	1.04±0.07	1.02±0.05			1.36±0.20	1.37±0.19		

[a]$P<0.05$, *vs* other groups; [c]$P<0.05$, *vs* groups 3 and 4. Student's unpaired *t* test.

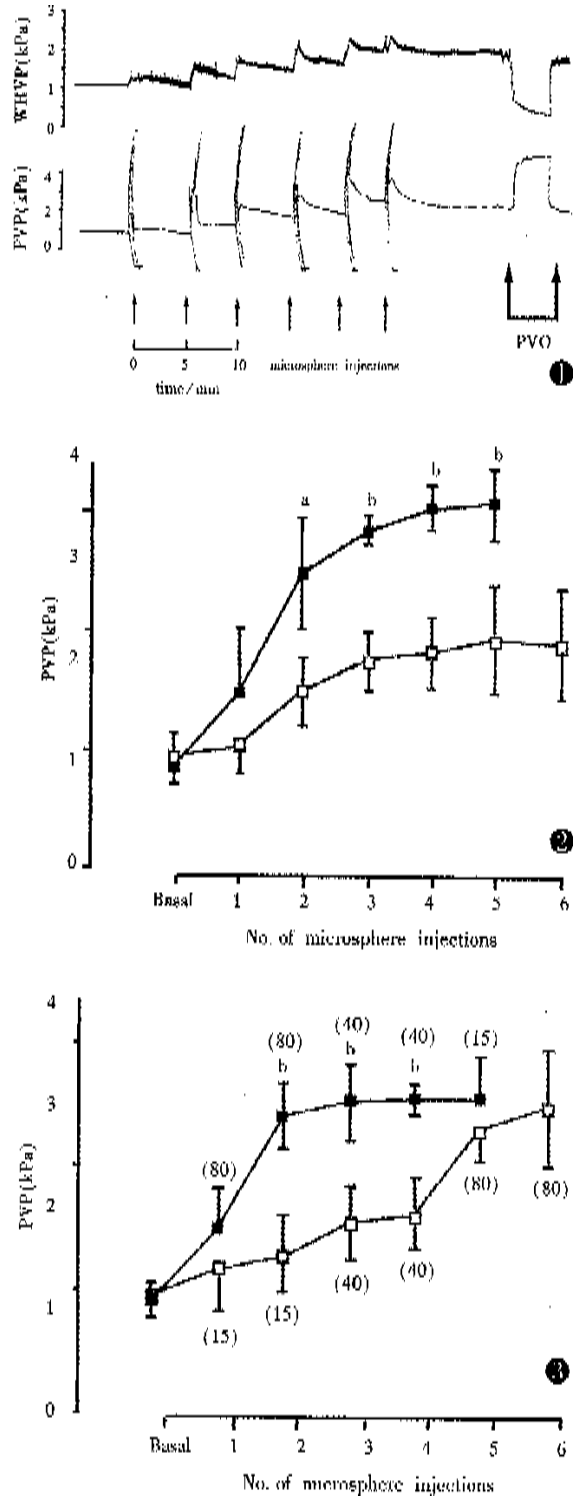

Figure 1 Portal venous pressure (PVP) and wedged hepatic venous pressure (WHVP) recordings obtained from a rat in Group 1 during intraportal microsphere injections and subsequent portal venous occlusion (PVO).

Figure 2 Changes in portal venous pressure (PVP) following intraportal microsphere injections in Group 1(□) and Group 2 (■).

[a]$P<0.05$, [b]$P<0.01$, Groups 2 *vs* Group 1.

Figure 3 Changes in portal venous pressure (PVP) following intraportal microsphere injections in Group 3(□) and Group 4 (■). Four rats in Group 4 died after the 6th injection and therefore steady PVP recordings could not be obtained after this time. [b]$P<0.01$, Group 4 *vs* Group 3.

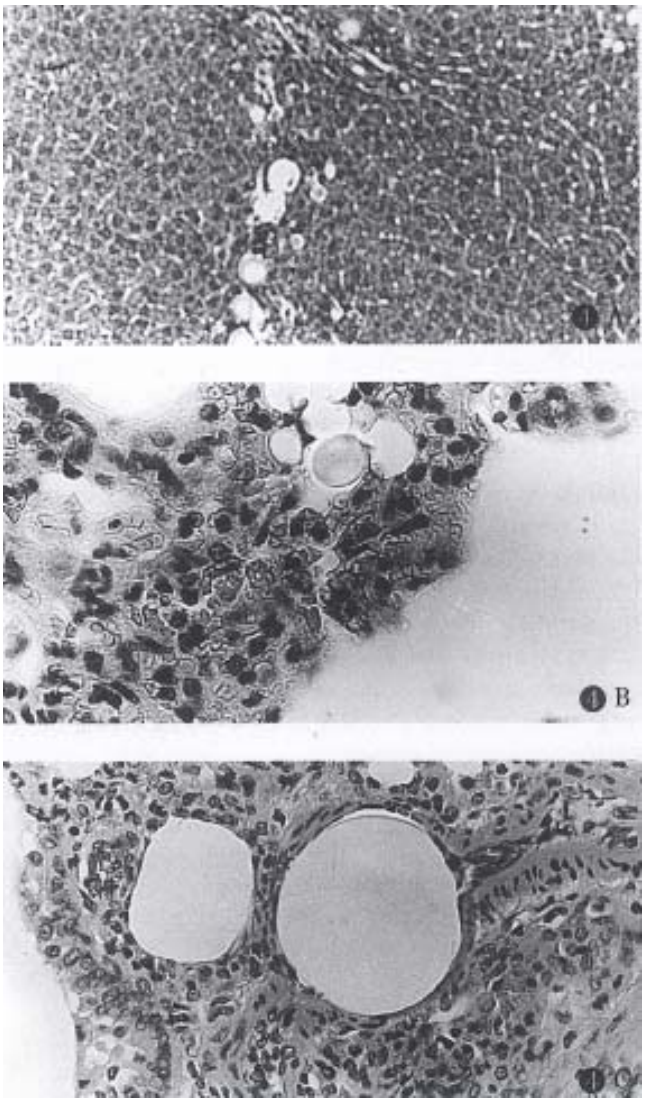

Figure 4 Histological sections showing (A) 15, 40 and 80 μm spheres lodged in liver (Group 3, original magnification × 10), (B) 15 μm spheres lodged in lung (Group 1, original magnification × 40) and (C) 80 μm spheres lodged in lung (Group 2, original magnification×16).

Histological examinations showed that in Groups 1 and 2 almost all of the portal radicles identified in the fields were blocked by the injected microspheres, with 15 μm spheres (Group 1) lodged in the terminal portal venules and 80 μm spheres (Group 2) lodged in the large portal radicles. In Group 3, the large and small portal radicles were simultaneously blocked by the different sized spheres injected. However, in Group 4, portal venules were mainly obstructed by the 80 μm spheres, while many 15 and 40 μm spheres injected after the 80 μm diameter spheres were trapped over the 80 μm spheres. The results in Group 5 were similar to Group 2, but with fewer spheres than those observed in Group 2. Numerous microspheres were found in the lungs of all experimental groups (Figure 4).

DISCUSSION

Anatomically the intrahepatic portal tract branches into progressively smaller radicles until the sinusoids are reached. Therefore, intraportal injection of microspheres can block the intrahepatic portal radicles and lead to presinusoidal portal hypertension. The present study confirmed that the PVP was elevated to a steady state 100% - 270% above the basal value following microsphere injections. Obviously, the increase in PVP was related to the size and numbers of microspheres used: the 80 μm spheres caused a large, rapid rise in PVP compared to 15 μm spheres (Figure 2). As shown by the histology, the 80 μm spheres were trapped in large portal radicles and therefore smaller numbers would produce a marked PVP raising effect. The results of sequential injections of different-sized microspheres showed that the final PVP was dependent upon the effect of the largest spheres (Figure 3).

When intrahepatic portal radicles were blocked by the microspheres injected, the presinusoidal increase in resistance produced not only an elevated PVP, but also a markedly lowered MAP because of extensive mesenteric pooling of portalve nous blood. Therefore, both the PVP and MAP should be considered into the reproduction of this model. From the results of the first four groups, it was found that two injections (1.8 × 105) of 80 μm spheres created an augmentation in PVP with the recovery of MAP. Furthermore, all rats in Group 5 showed a normal MAP and high PVP of 2.53 kPa ± 0.17 kPa which was comparable to the value of 2.75kPa ± 0.07kPa in the control group after partial portal venous ligation, and to the value of 1.8 kPa ± 0.2 kPa found in our cirrhotic model previously induced by carbon tetrachloride[2]. These suggest that 1.8 ± 105 of 80 μm spheres is the suitable dose for induction of portal hypertension in the model.

Clinically, portal hypertension is mainly caused by different forms of chronic liver disease, and is characterized by an increased resistance in the liver and by the formation of portosystemic collaterals. Though in early studies the site of the increased resistance was thought to be postsinusoidal, recent works have proved that the presinusoidal resistance is also increased in cirrhotic liver and contributes to the development of portal hypertension[5,6]. In particular, the presinusoidal block is the main cause of the idiopathic portal hypertension and the portal hypertension resulted from chronic biliary obstruction [7,8]. Therefore, this model, in regard to the location of increased intrahepatic resistance, possesses the general features of clinical cases. Moreover, the fact that many microspheres appeared in the pulmonary vascular bed suggested the opening of portosystemic collaterals during the procedure, with a diameter of more than 15 μm - 80 μm.

Originally we believed that the presence of microspheres in the lung is only due to the opened extrahepatic collaterals, and this seems to be supported by the observation that the SPP was slightly lower than PVP. However, in this study although the PVP in groups 1 and 2 did not increase significantly after the 3rd injection, which implied that the intrahepatic portal radicles had been saturated by the microspheres injected, the final PVP achieved was substantially less than that obtained by portal vein occlusion. Consideration of the simultane ous changes of PVP and WHVP in Group 1 strongly suggested the existence of intrahepatic portal-systemic shunts in the normal rat liver. Opening of these shunts would permit portal blood flowing directly into the hepatic veins, leading to an elevation of WHVP and preventing further increase in PVP. When the portalvein occlusion was performed extrahepatically, the function of intrahepatic shunts was deprived, and, as a result, PVP could rise to an extremely high level. The intrahepatic shunts have not been described in the normal liver, but have been reported in cirrhotic livers in rats[9] and in humans[10]. It has been suggested that the frequency of large shunts (diameter>25 μm) is relatively low and this is probably responsible for the reduction in WHVP after 80 μm microsphere injections in this study.

This model of portal hypertension is intrahepatic and can be induced rapidly, with the opening of intra- and extra-hepatic portal-systemic shunts. A major advantage of this model is that while the intrahepatic presinusoidal block is achieved acutely, the normal liver architecture remains. This may be particularly beneficial to the research in the actions of mechanical obstruction or some humoral substances, related to liver dysfunction[2], in the pathogenesis of portal hypertension. In the experimental cirrhotic model, it is difficult to differentiate these two actions.

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Original Articles

Tumor radioimmunoimaging of chimeric antibody in nude mice with hepatoma xenograft *

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Subject headings liver neoplasms, experimental; carcinoma, hepatocellular; chimeric antibody; mice, nude; hepatitis B virus; disease models, animal; radioimmunodetection; radioimmunotherapy

Abstract

AIM To study the radioimmunoimaging (RAII) using the human/mouse chimeric Ab to evaluate its targeting activity in animal models.

METHODS To chimeric Ab was labeled with ^{131}I. RAII was performed at different intervals after injection of radio-labeled Abs in nude mice with human hepatoma xenograft, and tissue distribution of radioactivity was measured. Comparison was made in the chimeric Ab between the single segment Ab and previous murine mAb against HBxAg.

RESULTS The experimental objects developed tumor-positive image after 2 days of radio-labeled Abs injection, and the peak accumulation of radioactivity fell on the 7th day. The tumor/liver ratioactivity of the chimeric Ab, single segment Ab, anti-HBx mAb, and the control group was 281±0.21, 2.44±0.16, 4.60 ±0.19, and 0.96±0.14, respectively.

CONCLUSION The genetic engineering Abs have a considerable targeting activity which can be used as a novel humanized vector in the targeting treatment of liver cancer.

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Patients with such a tumor can not, by and large, be diagnosed in its early stage and, therefore, lose the chance for operation. The purpose of this study was to explore the potential value of chimeric human/mouse Ab applied to the treatment of HCC in targeting therapy. Clinical evidence showed that the anti-HBx mAb prepared by HCC-related hepatitis virus X protein was effective vectors[1-3]. However, because of its murine-derived nature, the protein had certain immunogenecity, thus hindering its clinical applications in targeting therapy considerably. In order to circumvent the obstacle, modifications have been made to humanize the Abs using genetic engineering techniques. The light- and heavy-chain variable region (VH, VL) gene of the cloned anti-HBx mAb were ligated with the constant region (CH) gene of human IgG, resulting in the construction of a human/mouse chimeric Ab gene and itsexpression in the prokaryotic vector[4]. In this study, we carried out the radioimmunoimaging (RAII) on nude mice models bearing human HCC using ^{131} I-labeled chimeric Ab, aiming at characterizing the localization of the chimeric Ab in the HCC, and eva-luating its potential use as a vector in targeting therapy.

MATERIAL AND METHODS

Preparation of the chimeric human/mouse Ab and the single segment Ab

The chimeric Ab gene was cloned into pUC19, and subcloned into expressing plasmid PBLMVL$_2$, which was a gift from Professor Wu (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, China). A single colony from a fresh plate was incubated in a 3 ml culture and grown overnight at 30℃; then transferred into fresh medium, incubated at 30℃ for 2 h-3 h until A_{600} = 0.5, and then induced in a medium containing 15 mM $MgSO_4$ at 42℃ for 3 h. The bacteria were collected, cytolysed by ultrasonication, and aspirated. The supernatant was dried by vaccum and stored at -20℃.

Labeling of the Abs

The chimeric Ab, single segment Ab, anti-HBx mAb and expression protein of *E. coli* (TG1) were labeled with ^{131} I (provided by the Chinese Atomic Energy Institute) by the Iodogen method. Unincorporated ^{131}I was separated from the bound

iodine by gel filtration on a Sephadex G-50 column. The whole process of labeling was a bacteria free and pyrogen free event[5].

Animal model

22 LTNM4 nude mice models bearing human HCC by subcutaneous implantation were established in the authors' institute. Of these models, 5 mice were injected with chimeric Ab, 5 with single segment Ab, 5 with anti-HBx mAb as positive control, 5 with ^{131}I as negative control, and 2 with TG1. A small amount tumor sample (2 mm^3 - 3 mm^3) of HCC tissue originated from a nude mouse was transplanted subcutaneously into the 22 nude mice, and tumors measuring 1 cm in diameter were used for the immunoimaging study[6].

Radioimmunoimaging and biodistribution analysis

The experimental and control groups were injected intraperitoneally with ^{131}I-labeled human/mouse chimeric Ab, single segment Ab, anti-HBx mAbs, TG1 and ^{131}I respectively at a dose of 250 μCi per mouse. And γ imaging was operated on the 1st, 5th, and 7th day after injection. All animals were killed on the 7th day following the injection in order to determine the biodistribution of their radioactivity.

RESULTS

Immunoactivity assay of the radio-labeled Abs

The human/mouse chimeric Ab, single segment Ab, anti-HBx mAb, and TG1 were labeled by the Iodogen method. After separation of unincorporated ^{131}I from bound iodine, the binding activity of the chimeric Ab, single segment Ab, and anti-HBx mAb remained unchanged by ELISA (Figure 1).

Radioimmunoimaging of nude mice models bearing HCC

One day after injection of the ^{131}I-labeled human/mouse chimeric Ab and single segment Ab, the experimental animals showed distinct radio-accumulation in their abdominal cavities, whereas a faint -image was developed in tumor region. From the 5th day following injection, the distinct background image gradually disappeared, and an obvious radio-accumulation developed in tumor region. This phenomenon lasted until the 7th day. The positive control displayed tumor-positive image on the 5th day following injection of anti-HBx mAb, and even more clear tumor image was obtained on the 7th day which was in conformity with the previous reports. No accumulation was found in the negative control with injection of TG1 and ^{131}I. Since the thyroid glands of the nude mice were not blocked, they showed obvious radio-accumulation in the experiment (Figure 2).

Biodistribution of the radio-labeled Abs

Table 1 shows the radioactivity of tumor versus other tissues expressed as T/NT ratio following the injection of radio-labeled Abs after 7 days.

The tumor/liver ratio in human/mouse chimeric Ab and single segment Ab was 2.81 and 2.44, respectively in the experimental group, which was significantly higher than that of ^{131}I and TG1 in the negative control group (0.96 and 0.41). But they are lower than the tumor/liver ratio of murine derived mAb (4.6) in the positive control group. In addition, there has been a high accumulation of radioactivity in blood, gastrointestinal tract and kidney, especially predominant in the human/mouse chimeric Ab.

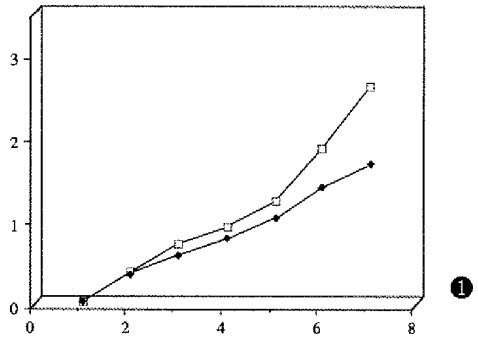

Figure 1 The binding activity curve of chimeric Ab by ELISA. MoAbs: anti-HBx mAb; sfv: anti-HBx human / mouse chimeric Ab

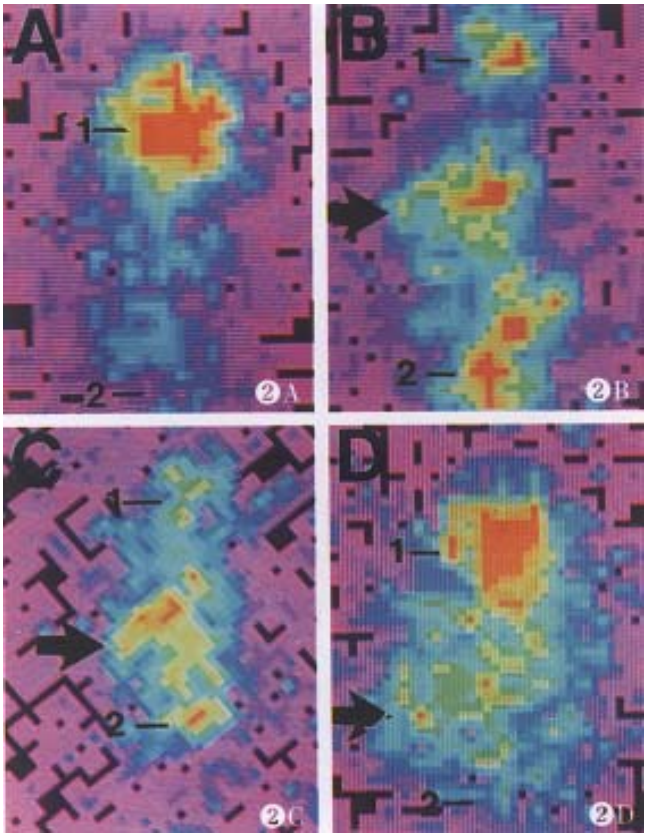

Figure 2 The localization of 131I-labeled chimeric Ab in nude mice bearing human HCC.
A: ^{131}I-TG1 B: ^{131}I-chimeric Ab
C: ^{131}I-single segment Ab D: ^{131}I-anti-HBx mAb
Arrow: the tumor image
1. The thyroid gland area 2. The bladder area

Table 1 T/NT ratio of 131I in nude mice models bearing human HCC ($\bar{x}\pm s$)

	^{131}I-labeled chimeric Ab	^{131}I-labeled segment Ab	^{131}I-labeled anti-HBx mAb	^{131}I	^{131}I-TG1
Blood	1.81±0.35	3.26±0.09	1.70±0.05	0.76±0.14	1.18
Liver	2.81±0.21	2.44±0.12	4.60±0.20	0.96±0.14	0.41
Kidney	0.48±0.09	2.22±0.07	2.66±0.15	0.42±0.05	0.69
Spleen	0.64±0.09	1.59±0.13	1.74±0.01	0.97±0.09	0.37
GI	1.06±0.03	0.40±0.08	2.67±0.33	0.90±0.01	0.07
Heart	3.84±0.37	3.23±0.23	6.10±0.51	1.86±0.15	2.39
Bone	4.25±0.20	5.71±0.11	6.70±0.24	1.84±0.13	1.97
Muscle	10.86±0.76	11.39±0.26	10.38±0.16	3.62±0.22	4.07

DISCUSSION

Targeting therapy has been applied to the clinical treatment of liver cancer as a powerful means to kill tumor cells for more than a decade. In this context, mAbs were usually the first choice. However, because of the murine origin of these mAbs, their repeated use often led to generation of anti-antibodies which would markedly reduce their bio-efficiency. Although several methods were adopted such as changing species and varieties of antibodies' origin, adding immunosuppressors, etc., the incidence of human anti-mouse Abs (HAMA) was 63% - 95% after the treatment with murine derived antibodies. The incidence of HAMA was 34.4% in our report[7]. The ultimate approach to solving this problem is, perhaps to reduce the immnogenicity of antibodies, that is to humanize the antibodies.

Based on our previous results, the expression level of X gene of hepatitis B virus was much higher than that of s gene and c gene, the T/NT ratio of anti-HBx- mAb approached 4.5 in the nude mice models, clinical targeting therapy trial confirmed that anti-HBx mAb was an exciting vector, and a humanized chimeric Ab gene had been established[4,5,8], we are trying to evaluate its potential value in the targeting therapy as a novel vector.

The results indicate that a distinct accumulation of tumor radioactivity developed on day 5 after injection of radio-labeled chimeric Ab, and an even more obvious accumulation was obtained on day 7. In bio-distribution analysis, the T/NT ratio on day 7 of the chimeric Ab, single segment Ab, positive control anti-HBx mAb, negative control TG1 and ^{131}I was 2.81, 2.44, 4.60, 0.41 and 0.96, respectively. This promising result suggests that recombinant Abs do have affinity to liver cancer tissues. Therefore, they can specifically direct against liver cancer tissues.

Although the chimeric Ab displayed certain high T/NT ratio, it did not surpass that of murine derived mAb. The reason behind this may be as follows: ①The Ab used in this experiment was the expression product of *E. coli*. When it was extracted from the bacteria by sonic cytosis, the extracts were none other than primary yields of *E. coli*. They contained not only the heat-induced Ab protein, but also other impure proteins derived from the expression of *E. coli* itself. For fear of possible inactivity of the Ab by gel filtration method, the expression product was not further separated and purified. This may account for the relatively low T/NT ratio in *E. coli*; ②The chimeric Ab was the product of prokaryotic expression vector. It is short of post-translational processing toward foreign protein by a eukaryotic system, such as glucolation, methylation, carboxylation, etc,. This may result in the production of an imperfect protein from which the experimental results may suffer.

In regard to the relatively high accumulation of radioactivity in blood, kidney, spleen, etc., the possible answer may be due to the impure Ab protein containing foreign substances which were apt to be uptaken by mononuclear cells, leading to the increase in the nonspecific binding.

The labeling ability was quite low for the chimeric Ab and single segment Ab in this experiment. Initially, we attributed this fact to fewer lysine groups of the recombinant Ab than those of murine derived mAb, for the principle of Iodogen method is to incorporate iodine with lysine groups of protein. Later, we found the amount of lysine groups of the genetic engineering Ab were no less than those of murine derived mAb. Therefore, our conclusion in terms of the low labeling ability of the recombinant Ab was the impurity of the protein with excess of foreign substances, and the lack of modification during post-translational processing. In a word, recombinant chimeric Ab has the advantages of low immunogenicity and high bio-efficiency of antibody-mediated ADCC and CDC, which certainly approve its qualification in clinical use. There is, however, much to be desired in terms of the practical use of chimeric Ab in clinical targeting therapy. Currently, work is underway in our institute to further separate and purify these chimeric Ab, make it expressed in eukaryotic systems in order to obtain intact secretory Ab.

In line with the advance of novel technologies in biomedical sciences, the increasing use of phage display antibody library technique, the antibodies, derived from the total antibody library, can be constructed *in vitro* without immunization *in vivo*. It can be well predicted that in the near future, certain active proteins with high affinity will be produced to benefit the mankind.

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Establishment of a nonradioactive assay for2′-5′ oligoadenylate synthetase and its application in chronic hepatitis C patients receiving interferon-α

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Subject headings 2′-5′ oligoadenylate synthetase/analysis; interferon-alpha; chromatography, thin layer; hepatitis C/enzymes

Abstract

AIM To establish a nonradioactive assay for 2′-5′ oligoadenylate synthetase (2-5 AS) and to measure the 2-5AS in peripheral blood mononuclear cell (PBMC) extracts of patients with chronic hepatitis C before IFN-α injection, 24 hours and one month after the first injection.

METHODS 2-5AS in cell extracts of PBMCs from 10 normal persons and 15 chronic hepatitis C patients were determined with PEI cellulose thin-layer chromatography.

RESULTS The assay of 2-5AS in human PBMC was found to be rapid, sensitive, specific and reliable. The 2-5AS activity of PBMC in normal persons was in a quite low level (2.0%), and it was increased about ten-folds after stimulation of IFN (19.7%), ($P < 0.01$). In 15 chronic hepatitic C patients, the basal levels of 2-5AS before IFN treatment were higher than those of normal persons, being much higher in the group showing poor response to IFN treatment, but 24h after the first injection of IFN-α the 2-5AS level showed a more rapid and much greater rise in those patients with a good response.

CONCLUSION 2-5AS may be a useful parameter of biological response during the IFN therapy.

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INTRODUCTION

The double-stranded RNA (dsRNA) dependent enzyme, (2′-5′) oligoadenylate synthetase (2-5AS), which was first discovered in interferon (IFN)-treated cells, could polymerize ATP into (2′-5′) oligoadenylate (2-5A). These were probably related to the antiviral activity and perhaps antiproliferative effects of IFN, called 2-5AS system. Treatment of cells (such as peripheral blood mononuclear cells, PBMC) with IFN led to the de novo synthesis of 2-5AS; this enzyme could produce oligoadenylates from ATP in the presence of double-stranded RNA (ds RNA) according to the equation: $(n + 1)\ ATP \rightarrow PPP5'A(2'P5'A)_{n+n}PPi\ \ (n \geqslant 1)$. (2′ -5′) oligoadenylates greater than the dimeric form could activate an endoribonuclease (RNase L) which was able to cleave mRNA, leading to aninhibition of viral protein synthesis[1].

Recently, some researchers determined 2-5AS level of PBMC in patients with chronic viral hepatitis as a biological response parameter during the therapy with IFN or in the diagnosis of diseases related to the IFN system. Now, lots of radioactive methods have been reported for measuring 2-5AS in EAT cells, Hela cells, Wish cells, rabbit reticulocytes and lymphocytes[2]. In this paper, we deve-lopeda non-radioactive assay for determining the 2-5AS in human PBMC: PEI-cellulose thin-layer chromatography.

MATERIALS AND METHODS

Preparation of rabbit reticulocyte lysate

Rabbit reticulocytes were prepared from adult male New Zealand rabbits (from Animal Laboratory of Beijing Medical University) by a modified method of Borsook *et al*[3]. One ml of a neutralized 2.5% aqueous solution of phenylhydrazine hydrochloride was injected subcutaneously each day, together with 0.05 mg of folic acid and 0.05γ of vitamin B_{12} were injected intramuscularly daily, reticulocyte counting was performed on smears of blood drawn from ear veins and stained with brilliant cresyl blue in 0.15mol/L NS. On the 7th or 8th day, blood samples were drawn while over 90% of the circulating red cells were then reticulocytes.

The blood samples were added to a flask containing heparin, washed three times with 0.01 mol/L phosphate buffer solution (PBS) pH 7.4. After centrifugation, the supernatant was removed

completely and the cells were treated with equal volume of lysis buffer (20 mmol/L Tris-HCl pH 7.6, 5 mmol/L magnesium acetate, 30 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, 10% glycerol, 0.5% Nonidet P-40) at 4℃ for 30 minutes. After centrifugation (15,000 × g, 10 minutes at 4℃), the 2-5 AS containing supernatant was collected and frozen at -70℃ for use.

Preparation of human PBMC extracts

Human PBMC of 10 normal persons were isolated from heparinized whole blood (5-10ml) by Ficoll-Hypaque sedimentation (Lymphocyte Separation Medium). Then 1×10^7 washed PBMC per ml were incubated 24 hours (37℃, 5%CO_2) in RPMI-1640 supplemented with 20% fetal calf serum with or without adding IFNα (INTRON, Schering, NJ) at 1000 U/L. After washing three times with PBS, PBMC were sedimented at 9000×g for 1 minute and then lysed by lysis buffer (1×10^7 cells per ml), and extracts were prepared by centrifugation of the lysed cells at 15 000×g for 10 minutes at 4℃, then were stored at -70℃ for use.

PBMC extracts from fifteen chronic hepatitis C patients were prepared as stated above, before IFN-α injection, 24 hours and also 1 month after the first injection of IFNα. They were stored at -70℃ for use.

Assay of 2-5AS

Because 2-5AS can catalyze the synthesis of 2-5A, the detection of 2-5A might be a reflection of the 2-5AS activity. In this study, we used the PEI-cellulose thin-layer plates (Sigma, USA) to separate 2-5A from residual ATP. The plate can specially separate the chemical compound with over 3 molecule-phosphate residues from those with less than 3 molecule-phosphate residues by thin-layer chromatography. First, the residual ATP not polyme-rized into 2-5A was digested with digestion buffer (Hexokinase 2.52 g/L, 30 mmol/L glucose monohydrate, 10 mmol/L magnesium acetate) to transform it into ADP (with 2 molecule-phosphate residues), then the chromatography was carried out, so the 2-5A was separated from ADP. The results of chromatography could be seen under ultraviolet lamp.

Poly (I) : poly (C) agarose beads were washed with buffer A (20 mmol/L Tris-HCl pH 7.6, 5 mmol/L magnesium acetate, 30 mmol/L 2-mercaptoethanol, 1mmol/L EDTA, 10% glycerol) three times, then suspended in buffer A (1 + 1 by vol) and 40 μl aliquots were transferred into Eppendorf tubes. After centrifugation and removal of supernatant, 400 μl PBMC extracts (or 0.8 ml rabbit reticulocytes lysates) were added into the tubes, reaction continued for 1 h at 4℃. Following this binding reaction, the agarose-beads were washed four times with 800 μl buffer A. The 2-5AS reaction was started by addition of 25 μl buffer B (buffer A + ATP 3 mmol/L, poly (I) : poly (C) 50 mg/L, creatine phosphokinase 0.1 g/L, creatine phosphate 6mmol/L), then incubated overnight at 37℃, the reaction was stopped by heating the mixtures (3-5 minutes at 95℃). After 2-5AS reaction, 20 μl of incubation mixture supernatants was transferred into wells of microtiter plates, 5 μl of digestion buffer were added (30 minutes at room temperature). Then all the samples were spotted onto a PEI-cellulose thin-layer plate (20 cm × 20 cm) 2 cm from the bottom. After drying the spots, the plate was immersed in methyl alcohol for 10 minutes with continuous shaking. After drying the plate, the chromatography was developed in chromatographic buffer (0.75 mol/L potassium phosphate tribasic anhydrous, pH 3.5) and developed to the top of the plate in this solvent, the procedure took about 10 minutes. The plate was then dried and exposed to ultravioletray: there was only one ultraviolet absorbing spot of "ADP" to the PBMC sample without 2-5AS activity, but there were two spots (one for "ADP"; another for "2-5A") to the sample with different 2-5AS level. Under UV analyzer, each spot was then cut out with scissors, to be immersed into 0.5 ml soaking buffer (20 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA, 1.5 mol/L NaCl) overnight, and absorbance at 259 nm was determined by the supernatant of each sample. The 2-5AS activity was calculated from the percentage of ATP turnover, according to the following formula:

$$\frac{OD_{2\text{-}5A}}{OD_{2\text{-}5A}+OD_{ADP}} \times 100\%$$

Study designs

Study 1: Each batch of rabbit reticulocyte lysate was subject to examination sixteen times.

Study 2: PBMC extracts from 10 normal persons were measured for 2-5AS with or without induction by IFNα, and the results were used for determining the varying responses in different normal persons to IFNα.

Study 3: Fifteen patients with chronic hepatitis C (11 male, 4 female; the average age for 40.7 years) who were treated with IFNα were studied. All the patients were positive for anti-HCV and HCV-RNA, but negative for anti-human immuno-deficiency virus (HIV) and anti-hepatitis A, B and D, their diseases had lasted more than one year, and all the fifteen patients had elevated serum alamine aminotransferase (ALT) activities of at least twice the upper limit of normal before IFNα therapy.

The patients were given IFNα intramuscularly for 3 months, at a dose of 3 mega units (MU) daily during the first 4 weeks and then thrice weekly for 8 weeks. Determinations for 2-5AS of PBMC were

performed in samples obtained pre-treatment, 24 h and 1 month after the first injection. At the same time, the fifteen patients' serum samples were examined every two weeks for ALT and anti-HCV, HCV-RNA. This study was to determine the relation between 2-5AS level and the curative effect to IFNα for chronic hepatitis C patients.

Statistical analysis

Statistical analysis was made with *t* test.

RESULTS

Digestive results of the residual ATP

To 25μl of the buffer B was added different volumes 0μl - 5μl per sample) of digestion buffer. After reaction, the incubated mixtures were spotted onto a plate and the chromatography was developed, the digestive percentage of ATP with different volumes of digestion buffer is shown in Table 1.

The digestive ratio of ATP was analyzed according to the similar formula of the turnover percentage of ATP:

$$\frac{OD_{ADP}}{OD_{ADP}+OD_{ATP}} \times 100\%$$

The results of Table 1 suggested that when 4μl of digestion buffer was added, the residual ATP in the incubation mixtures could be changed completely into ADP even if there was no 2-5AS activity in the sample. To ensure the accuracy of the assay, 5μl of digestion buffer was added.

Results of rabbit reticulocyte lysates (study 1)

The results of rabbit reticulocyte lysate in the 16 determinations of a single sample for 2-5AS were 31.6 ± 0.02 ($\bar{x}$%±s), with CV of 6.0%.

Results of 2-5AS in PBMC of 10 normal persons with or without induction by IFN-α (study 2)

PBMC extracts were examined for 2-5AS in one batch; the average levels of the cells before (Group A) and after (Group B) induction by IFNα are shown in Table 2.

Results of 2-5AS in 15 chronic hepatitis C patients (Study 3)

In the fifteen patients, after IFNα therapy, the ALT normalization rate was 53.5% (8/15) and the ALT reduction rate (> 50% decrease of baseline ALT) was 33.3% (5/15), with a total improvement rate (normalization plus reduction rate) of 86.6% (13/15); clearance rate of HCV-RNA was 60.6% (9/15) and of anti-HCV was 20.0% (3/15).

Before IFNα therapy, the 2-5AS average activity in PBMC of 15 patients was 12.6%, significantly higher than that of the normal group (P<0.01); the 2-5AS levels of patients 24 h and 1 month after the first injection was 64.9% and 40.4% respectively, an increase of 7.0 and 4.7 folds of that before IFNα injection.

These 15 patients were divided into two groups. In group A (9 patients), HCV-RNA disappeared after IFNα therapy, and group B (6 patients) HCV-RNA remained detectable after treatment. Their 2-5AS levels are shown in Table 3.

Table 1 The digestive results of ATP

The volume of digestion buffer (μl)	0	1	2	3	4	5
Digestive percentage of ATP (%)	0	3.1	18.3	34.1	100	100

Table 2 2-5AS levels in different groups

	Group A	Group B
	Before IFNα stimulation	After IFNα stimulation
n	10	10
x(%)	2.0	19.7
s	0.02	0.09
P	<0.01	

Table 3 2-5AS levels in groups A and B patients ($\bar{x}$±s)

Group	*n*	2-5AS level				
		pre[a]	24h[b]	24h / pre	1 month[c]	1 month / pre
A	9	9.3±4.7%	72.1±15.3%	9.4±4.3	42.3±8.2%	6.2±5.0
B	6	17.5±5.9%	54.1±14.2%	3.4±1.6	37.5±7.0%	2.4±0.8
P		<0.05	<0.05	<0.01	>0.05	>0.05

[a]pre: pre-treatment; [b]24 h: 24 h after first injection; [c]1 month: 1 month after first injection.

DISCUSSION

Chronic viral hepatitis is one of the major infectious diseases that endangers the public health seriously and thus is an important world wide problem. At present, there is no effective medication other than interferon (IFN). It is known that IFN has antiviral, antiproliferative, and immunoregulating properties, and its therapeutic effect depends to some extent on the dose, schedule and route of adiministration. So the determination of the optimal dose, schedule and route of administration is an important way to improve the response of patients treated with IFN. But the concentration of IFN in peripheral blood is very difficult to determine because of its low blood concentration and short half-life. Recently, some people determined the interferon-induced enzymes (such as 2'-5' oligoadenylate synthetase, 2'-5' oligoadenylate phosphodiesterase and protein kinase *et al*) as a biological response parameter of IFN[4] therapy.

Since the assay of 2-5AS was first reported in 1979, lots of methods had been introduced[5-8]. In this study, a new nonradioactive assay of 2-5AS was developed, the results of 2-5AS in rabbit reticulocyte lysates showed that this assay was a sensitive, rapid and specific method. The assay of

2-5AS in PBMC of 10 normal persons with or without IFN induction showed the following results: ①The 2-5AS activity of PBMC in normal person was at a quite low level (2.0%), and the individual difference was obvious (0% - 7.1%, $s = 0.02$); ② The 2-5AS level increased about ten-fold after stimulation of IFN (19.7%), and the difference of 2-5AS levels before and after IFN stimulation was significant ($P < 0.01$); ③ There was much differences in the response of IFN stimulation in different persons (9.5% - 36.6%, $s = 0.09$) and this might be a reason to explain a variable therapeutic effect of IFN in different patients.

Now, many reports have shown the relationship between 2-5AS level and the therapeutic effect of IFN in chronic hepatitis B patients[9,10], but we have not seen any report about chronic hepatitis C. In our study, the newly developed assay of 2-5AS (PEI-cellulose thin-layer chromatography) was used to determine 2-5AS in 15 chronic hepatitis C patients treated with IFN. The results showed that: ①The 2-5AS activity of PBMC in patients with chro-nic hepatitis C before treatment was much higher than that of normal persons (12.6% *vs* 2.0%, $P < 0.01$). In patients with effective treatment (HCV-RNA disappeared after IFN therapy), the 2-5AS activity before IFN therapy was lower than that in patients with ineffective treatment (HCV-RNA remained detectable after IFN therapy) (9.3% *vs* 17.5%, $P < 0.05$). This might be associated with the endogenous IFN system, which has already been activated in patients with poor response to IFN the-rapy; the use of large doses of external exogenous IFN might inhibit the activated host IFN system. ②Twenty-four after the first injection, the 2-5AS of all 15 patients increased in different degrees, compared with its basal levels (average 7.0 fold increase), but the 2-5AS level in patients with effective treatment increased more significantly than in patients with ineffective treatment (72.1% *vs* 54.1%, $P < 0.05$; 9.4 folds *vs* 3.4 folds, $P < 0.01$). This suggested that a greater increase of 2-5AS in PBMC 24 h after the first injection of IFN might be associated with the effectiveness of IFN therapy. ③One month after the first injection, 2-5AS level was also higher than its basel level (average 4.7 fold increase), but there was no difference between patients with good response and those without (42.3% *vs* 37.5%, $P > 0.05$; 6.2 fold *vs* 2.4 fold, $P > 0.05$).

In summary, the results of our study suggest that: ①The developed assay in this study (PEI-cellulose thin-layer chromatography) is a reliable way to determine 2-5AS of PBMC. ②In patients with chronic hepatitis C, a less elevated basal level of 2-5AS of PBMC before IFN therapy and a rapid and greater increase in 2-5AS 24 h after the first injection of IFN may anticipate a better response to IFN therapy.

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Simplified isolation and spheroidal aggregate culture of rat hepatocytes

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Subject headings liver cell; cell isolation; tissue culure; aggregates

Abstract

AIM To explore a simplified method for isolation of hepatocytes and establish a method of primary hepatocyte culture with more aggregates and longer persisence.

METHODS Wistar rat hepatocytes were isolated by a single extracorporeal two-step perfusion method, and the cells were seeded on poly-HEMA coated flasks and cultured with hormonally defined medium and gentle shaking at regular intervals.

RESULTS The total yield of isolating hepatocytes amounted to 10^8 cells for each rat liver with the viability of more than 90% in all isolations. Under the nonadherent environments, the cells were found to attach to each other and form multicellular aggregates rapidly, and the aggregates became spheroidal shape after two days in culture. The morphologic characteristics and albumin synthetic function of the multicellular spheroidal aggregates can be maintained for one month.

CONCLUSION The simple and reliable isolation as well as large scale and longer time culture of hepatocytes can be used for experiments in liver cell transplantation and bioartificial liver support system.

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INTRODUCTION

Hepatocyte culture, as an optimal model in vitro, plays an important role in the studies of liver disease[1]. However, the collagenase perfusion technique in situ can not be performed easily, the usual culture of hepatocytes, seeded on tissue culture dish under classical culture conditions can not adapt to large scale and high density culture and can not maintain liver-specific functions for longer time, thus limiting the development and application of hepatocytes culture. In this study, we used a simplified two-step perfusion method and some effective measures to isolate the rat hepatocytes in vitro and culture hepatocyte as spheroidal aggregates.

MATERIALS AND METHODS

Animals

Wistar rats weighing 150 g - 200 g, were provided by the Experimental Animal Centre of Third Military Medical College. The animals have received standard laboratory diet.

Cell isolation

Liver cells were isolated by an adaptation of the two-step perfusion method[2]. Briefly, the animals were anesthetized with barbital (30 mg/kg, b. w, intraperitoneally) and their livers were removed intact. The liver was first perfused in vitro via the portal vein with warmed (37℃) Ca^{2+} and Mg^{2+} free Hanks balanced salt solution at a flow rate of 5 - 8 ml/min for 10 - 15 ml/min, and then perfused with 0.05% collagenase (Sigma, Type IV) in the same solution supplemented with 5 mM $CaCl_2$ and 50 mM HEPES. The reperfusion with collagenase soultion lasted 20min at a rate of 5 ml/min at 37℃. After 10min of incubation (37℃) with gentle shaking, the suspension was filtered and hepatocytes were sedimented at 50 g for 3min.

Aggregate culture

Freshly isolated liver cells (4×10^5 in 1 ml of RPMI 1640 medium) were seeded in culture flasks precoated with poly (2-hydroxyethyl methacrylate) (poly-HEMA, Sigma) and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. In order to inhibit cells attachment on wells and promote cells agregation, the culture flasks were rotated at a re-gular interval. After 4 h of incubation, RPMI 1640 medium was changed by a hormone defined medium (HDM) composed of Williams medium E supplemented with 10 mg/L insulin, 10 μg/L HGF, 50 μg/L EGF, 100 μg/L

hydrocortisone, 100 μg/L glucagon, 50 mg/L linoleic acid, 100 U penicillin, 0.1 mg/ml streptomycin, 1 mg/L amphotercin B and 10% FCS (mainly reagents were purchased from Sigma). The medium was changed every day.

Histology

Cell morphology in the culture was observed under Olympus phase contrast microscope. For electron microscopy the spheroidal aggregates of hepatocytes cultured for different time periods were fixed at 4℃ for 4 h with 3% glutaraldehyde in 0.1 M phosphate buffer and post-fixed for 1 h with 1% OsO_4, dehydrated in graded ethanol solutin and embedded in Epon 618. Ultrathin sections were prepared coventionally and observed with a JEM-2000SX transmission electron microscopy (TEM). Scanning electron microscopy (SEM) sample was dehydrated by the same method and further dehydrated by critical point-drying. A thin-layer of gold was deposited on the cell surface by a sputtering system and examined in AMRAY 1000B SEM.

Albumin production

The albumin synthesis by hepatocytes in culture media was determined on the Beckman CX-7 Synchron Clinical System.

RESULTS

The total yield of isolated hepatocytes by the simplified two-step perfusion method amounted to 2 - 4 × 108 cells for each liver. The viability of hepatocytes as judged by the trypan blue test was estimated as 90% - 98% in all isolations.

Under the defined culture environment and repeated gentle shaking, the freshly isolated hepatocytes attached to each other within 4 h - 8 h and multiple aggregates of different sizes were loosely formed and the multiplicity of aggregates increased along with time. Up to 48 h, a lot of regular spheroidal aggregates were seen in flasks. The aggregate hepatocytes spheroids appeared very tight and dense in centre, and on the surface of the spheroids attached a lot of single cells as flower (Figures 1,2). Through action of single cells, some spheroids were also attached to each other. The characteristics of spheroidal aggregates can be maintained until the 30th day of culture. At the same time, TEM revealed that the cultured hepatocytes had a large round nucleus and abundant cytoplasmic organelles as observed in normal ones (Figure 3).

When the aggregate culture stopped, cell spheroids were transferred into collagen-coated wells. After 24h of incubation (37℃, 5% CO_2) approximately, 74% - 81% spheroids were seen to attach on substratum and many cells incorporated into spheroids migrated out forming a monolary (Figure 4).

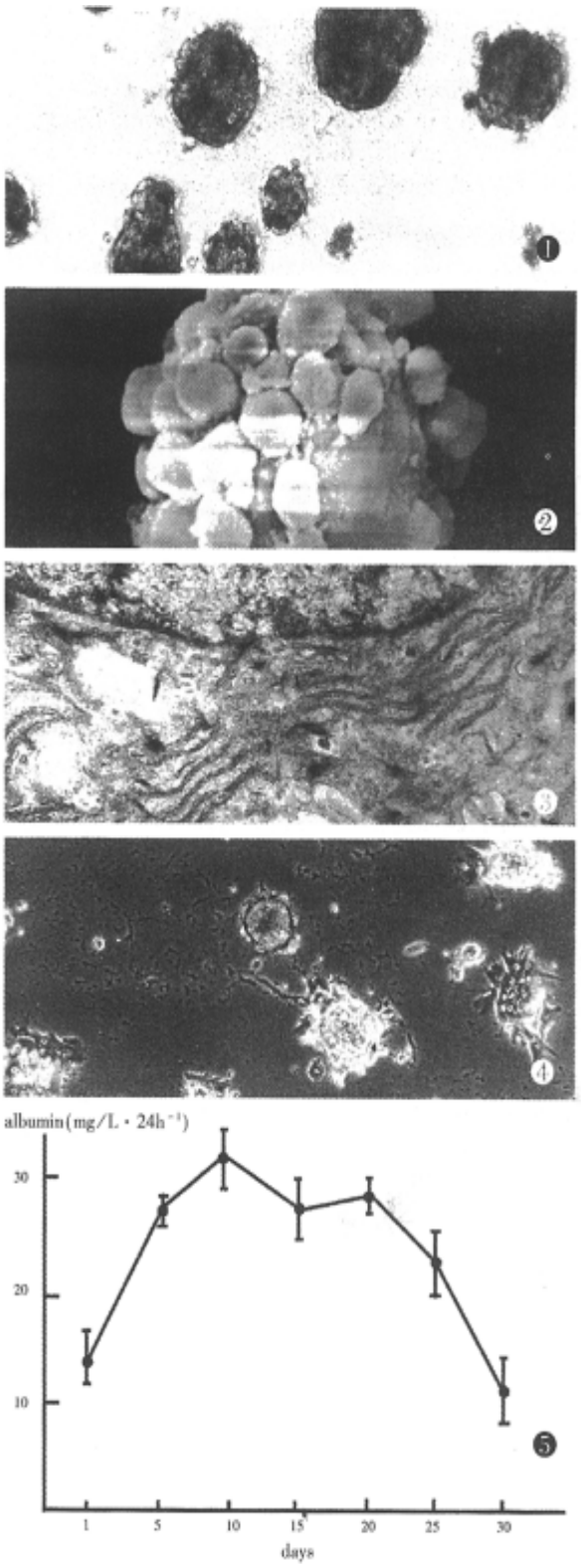

Figure 1 Phase-contrast microscopic feature of multicellular spheroidal aggregates of hepatocytes at day 4. (× 200)
Figure 2 Scanning electron microscopy of spheroidal aggregates at day 14. (× 1000)
Figure 3 Transmisson electron microscopy of hepatocyte spheroids cultured for 25 days, showing abundant organelles in hepatocytes. (× 10 000)
Figure 4 Phase-contrast photomicrograph of hepatocyte spheroids attached to collagen-coated wells. (× 100)
Figure 5 Albumin synthesis and secretion by aggregate hepatocytes.

Secretion of albumin by hepatocytes was virtually detectable in the aggregate culture. By 5 days in culture, a marked increase in albumin production was seen, which can be maintained until the 25th day of culture. By 30 days, albumin secretion decreased rapidly (Figure 5).

DISCUSSION

Until recently, hepatocytes are isolated according to the two-step perfusion method devised by Seglen. But as the method required a special apparatus and a lot of collagenase for liver perfusion in situ it is not so easy to completely dissociate the hepatocytes and to perform hepatocytes culture in ordinary laboratories. In our experiment, the rat liver was removed and perfused with colla genase in vitro. Although the yield of isolated hepatocytes is relatively low, the number still amounted to 108 cells and more than 90% of hepatocytes were viable. The equipment necessary for use was simple, the dose of collagenase was much lower than by Seglen method, and it can be adjusted according to the requirements of hepatocytes. These denote that the collagenase perfusion in vitro is a simple and reliable method for isolating hepatocytes from the biopsied samples and animal livers, which may be useful in performing hepatocytes culture widely.

Under the standard monolayer culture conditions, maintenance of survival and different functions of cultured hepatocytes have proven to be difficult. It is also difficult to provide large scale production of hepatocytes for some special use. Therefore some attempts have been made to increase the functional longevity and large scale production of hepatocytes in primary culture. For example, cultures of rat hepatocytes on Biosilon microcarriers allow one to obtain large metabolic active cells[3], cocultures of human or rat hepatocytes with rat liver epithelial cells allow the maintenance of several liver functions up to 2 months[4].

But the microcarrier method and coculture are unfavourable in use. Since the culture of neonatal rat liver cells in spheroidal aggregates was described by Landry *et al*[5], special attention has been paid to the culture method. Because the spheroids were formed in three-dimensional structures where cell-cell contacts are maximized, and the reaggregate and re-form structures resembled in some aspects of those found in vivo. Under those cultural conditions, the hepatocytes can mainta in viability and functional integrity for months. Our experimental results were in agreement with that, albumin was still secreted by cell aggregates up to 30 days of culture, at the same time, the cells were still capable of attaching collagen-coated wells possibly due to the effective methods including poly-HEMA, HDM, cell growth factors and a rotating method.

Culturing of hepatocytes as spheroidal aggregates may be preferable to monolayer culture as an experimental liver model. The exciting potential applications may include the use of these hepatic spheroids for investigation in therapeutic measures for acute hepatic failure, such as hepatocytes transplantation and the construction of extracorporeal bioartificial liver support systems.

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Effect of aging on cytoskeleton system of Kupffer cell and its phagocytic capacity *

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Subject headings Aging; Kupffer cell; cytoskeleton; phagocytosis

Abstract

AIM To investigate the age-related alterations of cytoskeleton system in liver Kupffer cell and their relation to the changed phagocytic function.

METHODS The phagocytic function of Kupffer cells from rats of various ages (6 mo, 12 mo,18 mo and 24 mo) were quantitatively evaluated by phagocytosis of polystyrene beads. The actin distribution and measurement of Kupffer cell were determined by a phalloidin-TRITC method; and the myosin and vimentin distribution and measurement with indirect immunochemical staining.

RESULTS Aging resulted in significant alterations of actin, myosin and vimentin distributions and reductions in Kupffer cell; the 3 cytoskeleton components of 24 -mo-old Kupffer cell were significantly decreased to 68.0%, 84.9% and 75.5%, respectively of these of 6-mo-old Kupffer cell($P < 0.01$,0.01 and 0.01). And these decreases had significant positive relations with the damaged phagocytosis of the aged Kupffer cell. γ values were 0.96($P < 0.05$), 0.99($P < 0.01$) and 0.95 ($P < 0.05$) respectively.

CONCLUSION The cytoskeleton system of the aged Kupffer cell presents an evident state of senescence, which may be an important mechanism of decreased phagocytosis of the aged Kupffer cell.

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INTRODUCTION

Kupffer cells account for about 30% of nonparenchymal liver cells and constitute the largest pool of resident macrophages in the body. They play an essential role in the elimination of foreign substance derived from the systemic circulation mainly through phagocytosis. The age-related alterations in Kupffer cell function are considered to be related to the susceptibility to sepsis after trauma or infection or to tumor of the old people[1]. In this study, the distribution and contents of actin, myosin and vimentin in Kupffer cells of various ages, and a quantitative evaluation of phagocytosis of polystyrene beads by primary cultured Kupffer cells of various ages will be described.

MATERIALS AND METHODS

Animals and groups

Twenty-four Wistar rats of different ages obtained from the Chinese Herb Research Institute of Sichuan Province were rendered into 4 groups (6, 12,18 and 24 months of age). Each group had 6 rats.

Kupffer cell isolation and culture

Liver nonparenchymal cells were isolated by a collagenase-perfusion method as reported previously[2]. Briefly, after anesthesia (30 mg of barbital/kg body wt., intraperitoneally), the liver was perfused *in situ* with Ca^{++} -free Hanks balanced salt solution at 37℃ for 3min. Then 0.05% collagenase (Sigma, Type IV) was added and the liver was perfused for a further 4min with Hanks balanced salt solution. After gentle shaking, the suspension was filtered and hepatocytes were sedimented at 50 × g for 3min. Non-parenchymal cells from the treatment were collected and sedimented at 300 × g for 10min. The pellet of nonparenchymal cells was resuspended and cultured with RPMI 1640 medium (containing 15 mmol/L HEPES, 0.05 U/ml insulin, 15 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% newborn calf serum. After 30min, the non-adherent cells were deleted. The viability of the KC was greater than 90% as determined by trypan blue exclusion.

Distributions and content measurements of actin, myosin and vimentin of Kupffer cell

Kupffer cells were fixed on a plastic dish and the phalloidin-TRITC (Sigma) method was used for

staining actin by a previously reported method[3]. The samples were observed under fluorescence microscope (Olympus VANOX) and the actin contents were determined using a fluorometer (Hitachi MPF-4). Indirect immunochemical staining was performed for myosin and vimentin using anti-myosin antibody and anti-vimentin antibody(Sigma)[4]. The samples were observed by using a 40-fold objective (Olympus VANOX). The staining images were collected by means of a Panasonic CL320 videotape camera and sent into an image analyzing computer system (CMIAS007) for quantitative assessment of the gray scale.

Quantitative analysis of phagocytic activity

The phagocytic function of Kupffer cell was quantitatively measured by a method previously reported[4,5]. Cultured Kupffer cells were incubated with RPMI 1640 medium supplemented with 10% fetal calf serum at 37℃ for 6 h. After the incubation, 1.7×10^8 polystyrene beads/dish (diameter 1.1 μm, Sigma) were added to the cultures, which were maintained for a further 60min at 37℃. The cultures were washed three times with RPMI 1640 medium and the numbers of beads in Kupffer cells were counted under an inverted phase contrast microscope (XSJ-D), Chongqing Optical Electric Appliances Plant). One hundred Kupffer cells randomly selected from each of 5 different cell preparations were used for the phagocytosis study.

Statistical analyses

The results were expressed as $\bar{x} \pm s$ and statistical analyses were made with one-way analysis of variance and Student's *t* test.

RESULTS

Distribution of actin, myosin and vimentin in rat Kupffer cells at various ages

Aging caused morphological changes in cytoskeleton system of Kupffer cells are shown in Figure 1. Kupffer cells of 6-mo-old rat appeared flat and there were several pseudopodia on the surface. The myosin was shown in the pseudopodia. In fluorescent staining for actin, intense specific fluorescence was observed in both the cytoplasm and the peripheral region along pseudopodia. The distribution of actin was similar to that of myosin. Vimentin positive fibres extended throughout the whole cytoplasm and a perinuclear accumulation was also present in 6-month-old Kupffer cells. Kupffer cell of 24-month-old rat appeared small and circular and the number of pseudopodia decreased. Weak and diffuse fluorescence and staining could be observed around the pseudopodia and the nuclei of the aged cells in actin and myosin staining respectively. Weak staining could also be observed throughout the cytoplasm of the aged Kupffer cells in vimentin staining.

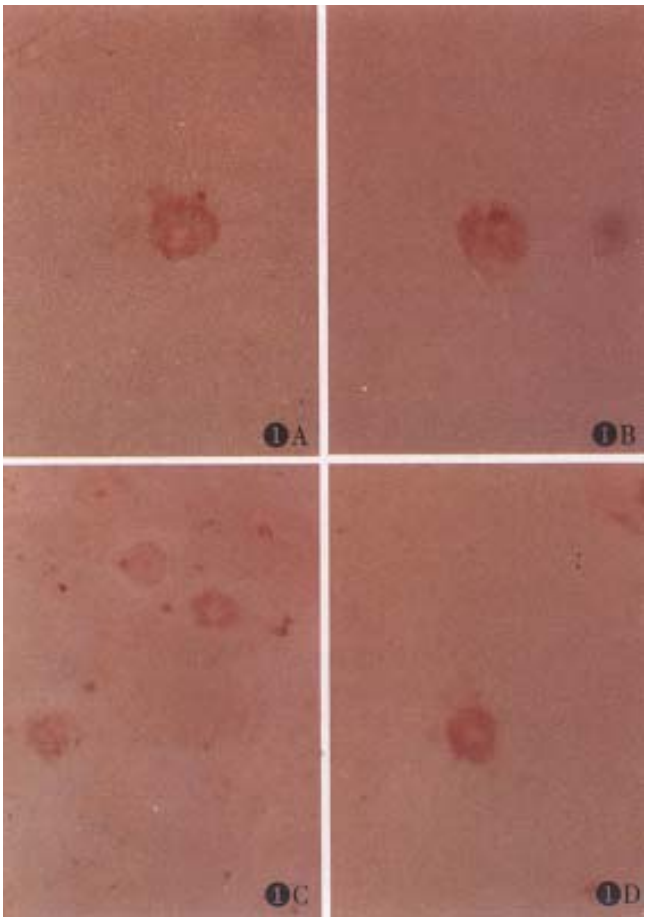

Figure 1 Distribution of myosin and vimentin of 6-month-old (a,b) and 24-mo-old(c,d) Kupffer cells (ABC×400).
a and c: myosin;b and d: vimentin.

Table 1 The actin, myosin and vimentin contents of Kupffer cells of each age group ($\bar{x} \pm s$)

Group	Actin (n = 6) (ng phalloidin/μg prot.)	Myosin (n = 5) (gray scale)	Vimentin (n = 5) (gray scale)
6 mo	8.63±0.60	134.88±8.14	137.50±5.71
12 mo	8.24±0.53	141.46±5.18	129.91±7.01
18 mo	6.20±0.71[b]	121.89±7.52[a]	118.13±13.54[a]
24 mo	5.87±0.49[b]	114.45±5.09[b]	103.08±8.41[b]

[a]P<0.05,[b]P<0.01, *vs* 6 mo.

Quantitative measurement of the 3 cytoskeleton components of Kupffer cells at various ages

Aging resulted in significant reductions of the 3 cytoskeleton components mentioned above, i. e., the contents of actin, myosin and vimentin in 24-mo-old KC were significantly decreased to 68.0%, 84.9% and 75.5% respectively of those in 6-mo-old KC (P<0.01,0.01 and 0.01), (Table 1).

Phagocytic activities of Kupffer cells from each group

In the 6-mo group, 19.6 ± 2.1 beads were taken up by cultured Kupffer cells during the 60min observation period. In 12-mo, 18-mo and 24-mo

groups, 20.8 ± 2.1, 12.7 ± 1.5, 8.6 ± 2.3 beads were taken up into Kupffer cells respectively. The difference between 6-month-old group and 12-mo-old group was not significant ($P > 0.05$). The difference between 6-mo-old group and 18-mo-old group and that between 6-mo-old group and 24-mo-old group were significant ($P<0.05$).

Analysis of correlation

Significant positive correlation was found between the changes of actin, myosin and vimentin contents in Kupffer cell aging and the damaged phagocytosis. γ values were 0.96 ($P<0.05$), 0.99($P<0.01$) and 0.95($P<0.05$), respectively.

DISCUSSION

Kupffer cell is the main component of the host monocyte-macrophage system. It is crucially important for the host to fight against infection or sepsis[6]. The previous study showed that the decreased phagocytosis of the aged Kupffer cell was responsible for the increased severity of pathophysiological changes after endotoxemia[7]. So it is of significance to study the mechanism of decreased phagocytosis in Kupffer cell aging.

A 24-mo-old rat was used in the present experiment as the aging model, which was comparable to the age of 65 - 75 in the human being, an age period consistent with the standard of old people of our country. So 24-mo-old rat may serve as a qualified model for the study of Kupffer cell aging[2].

The mechanism of phagocytosis by Kupffer cells is still not completely understood. The ruffling of cell membrane and formation of pseudopodia play an important role in the phagocytosis of Kupffer cells and this is believed to be accomplished by the cytoskeleton. In the cytoskeleton, actin-myosin interaction through the calcium-calmodulin systems plays a major role in this activity[8]. In this system, intracellular Ca^{2+} combined with calmodulin to form the active calcium-calmodulin complex, which activates an enzyme, myosin light chain kinase, for phosphorylating the light chain of myosin. Phosphorylated myosin, but not unphosphorylated myosin, can interact with actin to induce the activity of cell membrane and pseudopodia and then phagocytosis. The process of this system is reversible, in that a phosphatase can catalyze dephosphorylation of myosin, restoring it to a form that can not be activated by actin.

Vimentin is another important cellular cytoskeleton component. It is in radial arrangement in cytoplasm, forming a frame to support the actin-myosin system and other organelles. It can prevent the cell from being injured by changing its tension, thus keeping the cellular shape.

In the current study, the distribution and determination of actin, myosin and vimentin in Kupffer cells at various ages were studied and our observations reve aled that the distributions of the 3 cytoskeletons dramatically changed and their contents significantly decreased in aged Kupffer cells. The actin fluorescence and myosin staining in the area of the pseudopodia became weak in the aging Kupffer cells. The vimentin staining throughout the whole cytoplasm became weak too. These results indicate that the age-related damage of the cytoskeleton system in Kupffer cell aging is one of the important mechanism responsible for a decre ase in phagocytosis.

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Pancreatic tumor: DSA diagnosis and treatment

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Subject headings pancreatic neoplasms/ diagnosis; pancreatic neoplasms/therapy; angiography, digital subtraction

Abstract

AIM To study the clinical significance of the diagnosis and catheterized interventional treatment of digital subtraction angiography (DSA) for pancreatic tumors.

METHODS Ninety-two patients with pancreatic tumor, 69 males and 23 females, aged from 41 to 70 years (mean 57.1 years) were diagnosed with DSA. Sixty-one patients with pancreatic cancer were treated with transcatheter celiac and superior mesenteric arterial anticancer agents (MMC 20mg, EADM 40 mg and 5-FU 2.0 g) infusion (TCSAI).

RESULTS The DSA diagnoses were confirmed by operations and pathological examinations, with a coincidence 82.6% rate of, and a therapeutic effective rate of 42.6%.

CONCLUSION DSA is of diagnostic value for pancreatic tumors, and helpful in understanding the course of the disease, judging the prognosis and selecting the therapeutic regimen, and could improve the chemotherapeutic effect as well.

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INTRODUCTION

In order to study the clinical significance of the diagnosis and catheterized interventional treatment of digital subtraction angiography (DSA) for pancreatic tumors, 92 cases of pancreatic tumor were diagnosed with DSA, and 61 cases of pancreatic cancer were treated with transcatheter celiac and superior mesenteric arterial infusion of anticancer agents (TCSAI) from Nov. 1987 to April 1997, with sa-tisfactory results.

MATERIALS AND METHODS

Patients

Ninety-two patients (69 men, 23 women, aged from 41 - 70 years, averaging 57.1 years), were diagnosed as having pancreatic tumor through history of disease, B-ultrasonography, PTC and/or ERCP and CT.

Methods

Abdominal aortography was performed in the 92 cases 8 times, selective celiac arteriography 18 times, combined angiographies of superior mesenteric artery 22 times, common hepatic artery 22 times, proper hepatic artery 15 times and gastroduodenal artery 11 times (Siemens Angiotron-CMP Bicor DSA System) by percutaneous trans-femoral-artery catheters in Seldinger's way, 10 ml - 50 ml 76% meglucamine diatrizoate for each case. Among them, 61 cases of pancreatic cancer were treated with TCSAI for a total of 93 times. The anticancer agents (MMC 20 mg - 40 mg, FADM 40 mg, 5-Fu 2 g) were divided into 2 equal parts, and infused into the celiac artery and superior mesenteric artery, respectively. The cases of early and intermediate cancer were infused after resection and the cases of late and recurrent cancer were infused immediately after arteriography. In accordance with the patient's condition and curative effect, TCSAT was performed once every 3-4 weeks, but not more than 3 times in 61 patients.

RESULTS

Diagnosis

DSA diagnoses of 92 cases were confirmed by operations and pathological examinations. Among them, 89 cases had pancreatic cancer, which was located in the head of pancreas in 67 cases, in body of pancreas, 18 cases and in tail, 4. Thirty-nine were complicated with metastatic hepatic cancer, 21 had metastatic celiac cancer and 3 cases had pancreatic cystadenocarcinoma. The diagnostic coincidence rate was 82.6% (76/92), without

positive misdiagnosis rate, and the negative rate was 17.4%(16/92), which may be caused by less blood supply and underdevelopment. DSA examination had no obvious adverse reactions.

Treatment

The therapeutic effective rate (including the cases whose subjective symptoms were improved) was 42.6% (26/61). Tumors were either put under control or reduced in size in 31.1%(19/61). In 39 advanced cases of complicated with metastatic hepatic cancer, the 1-year survival rate was 51.3% (20/39), 3-year survival rate, 15.4% (6/39), and one patient died of late failure within 30 days. The main adverse reactions of TCSAI were nausea, vomiting anorexia, abdominal distention and so on, which usually disappeared in 1-3 days, and WBC decreased temporarily in 1-2 weeks. No severe complications happened.

DISCUSSION

To define the relationship between cancer and branches of gastroduodenal, hepatic artery, superior mesenteric vessels, portal vein and abdominal aorta is the key to decide whether to perform radical resection or enlarging resection (including superior mesenteric vessels resection). The other examinations used now for pancreatic tumors include B-ultrasonography, hypotonic duodenography, PTC, ERCP, CT and so on. However, B-ultrasonography can be disturbed easily by intra-abdominal gas and obesity hypotonic duodenography, PTC and ERCP can not show the range and diffusion of pancreatic cancer and relation of the tumor to peripheral vessels and CT can reveal involved vessels, but only the cross sections and not the whole length, while DSA was of the peculiar diagnostic value, which could not be substituted by the other examinations, in understanding the relationship between cancer and vessels.

Although DSA is more advanced than common angiographies, DSA originally does not belong to selective angiography[1], so that the crisscross and overlap of the vascular images may often hinder the diagnosis when there are many vessels in the examined size at the same time. In order to remedy this defect, we have improved the contrast techniques and methods to combine DSA with selective angiography so as to greatly raise the image quality and spatial resolution. That DSA is applied to celiac, superior mesenteric, hepatic and gastroduodenal arteriographies can continuously show the arterial, capillary and venous phases of the tumors in turn, so as to know size, shape, infiltrative range, metastasis and vascularity of the cancer, and further define the pathologic quality, disease course and assist in the cocalization of cancers.

Pancreatic cancers belong to ischemic tumors, therefore DSA arterial phase is the most important in the diagnosis of the cancer. Capillary phase and venous phase are of the significance of assistant diagnosis, the arterial phase mainly shows abnormal flexion, irregular dilatation, plexiform aggregation or interruption of gastroduodenal artery, superior and inferior pancreaticoduodenal arteries caused by compression and erosion of the cancer. In early stage, the appearance of gastroduodenal artery is normal or mildly stiff and displaced. In advanced stage, cancer infiltrates peripheral arteries, even hepatic artery and its branches, through pancreatic capsules. Carcinoma of body and tail of pancreas usually infiltrates superior mesenteric artery and splenic artery so that they can be irregular, flexional, stiff, constrictive or interrupted. Capillary phase usually shows filling defect in the site of cancer when pancreatic parenchyma is stained. Normal pancreatic head has so rich blood circulation that the staining is deeper in the head than in the body and tail. Early carcinoma of pancreatic head makes the staining of the head light. In late stage the staining disappear. Venous phase can show that portal vein, superior mesenteric vein and splenic vein are involved in advanced cancers. Pancreatic cystadenoma is also an ischemic lesion, the peripheral blood vessels are compressed and displaced outward and dilated, without the signs of stiffness, constriction and interruption, and the tumor margin is clear.

DSA high-quality images greatly raise the accuracy, safety and chemotherapeutic effect of TCSAI. Higher concentration anticancer agents act directly on the cancer and the lymph nodes to produce cytotoxins, block the synthesis of DNA in cancer cells and reduce the ineffective distribution of the agents in general normal tissues. The infusion can also give a better therapeutic effect to hepatic metastasis[2], resulting in inhibiting cancer growth, metastasis and recurrence, and increasing the possibility of resection.

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Bile acids in serum and bile of patients with cholesterol gallstone

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Subject headings cholesterol gallstone; bile acids/analysis; deoxycholic acids/analysis; chromatography, gas

Abstract

AIM To analyze serum bile acids and biliary lipids of patients with cholesterol gallstone(CS) and explore the relationship between deoxycholic acid (DCA) and CS disease.

METHODS Analysis of bile acids in serum was done with gas-chromatography in two groups: CS group (n = 151) and control group (n = 256). Serum bile acids and biliary lipids were also studied in 90 matched samples.

RESULTS The serum DCA was 0.955 μmol/L ± 0.078 μmol/L in CS group, which was more than that of control group (0.696 μmol/L ± 0.047 μmol/L), $P < 0.01$. The ratio of DCA/chenodeoxycholic acids (CDCA) was 1.76 ± 0.30 in CS group, about two times that in control group (0.92 ± 0.14). The mole percent of DCA in bile was positively related to cholesterol saturation index (CSI) ($P<0.01$) and the mole percent of CDCA in bile negatively to CSI ($P = 0.01$). There was correlation between the mole percent of DCA, CDCA and cholic acid in bile and in serum.

CONCLUSION It is suggested that DCA is lithogenic and the increased amount of DCA or the ratio of DCA/CDCA in serum may be one of the features of cholesterol gallstone patients.

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INTRODUCTION

Bile acids may play a role in the formation and dissolution of cholesterol gallstones. Chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) are litholytic bile acids, which can dissolve cholesterol fraction of gallstones and used in prevention. Deoxycholic acid (DCA), however, as one of lithogenic bile acids has attracted great attention[1]. The purpose of this study was to analyze bile acids in both serum and bile and biliary lipids in cholesterol gallstone patients, and find out the relationship between bile acids and biliary lipids, especially between DCA and biliary lipids.

MATERIALS AND METHODS

Subjects

Two groups were studied The cholesterol gallstone group had 151 patients. Among them 88 had cholesterol gallstones classified by both the observation of the cross section of stones obtained during cholecystectomy and chemical analysis of their components. The other 63 asymptomatic patients had radiolucent gallstones and well-functioning gallbladders shown by oral cholecystography. The control group included 247 healthy individuals by both physical examination and B-ultrasonography and 9 patients without gallstones confirmed at the time of non-biliary surgery. None of the subjects had any evidence of hepatic or renal abnormality, or other diseases such as diabetes, hypertension and coronary heart disease. Both gallbladder bile and venous blood were obtained during elective cholecystectomy. For the nonoperative subjects, only fasting blood was available.

Study protocol

In analysis of serum bile acids, the gallstone group comprised 63 males and 88 females, with an age range of 52.6 ± 11.5 years, and the control group 169 males and 87 females, with an age range of 50.0 ± 10.3 years.

In analysis of matched serum and biliary bile acids, all the subjects had undertaken abdominal operation. Eighty-one with cholecystectomy had cholesterol gallstones and 9 with non-biliary operations had no stone in the gallbladder, including 37 males and 53 females with a mean age of 50.5 ± 11.0 years.

Assay of bile acids

Bile samples were directly hydrolyzed with NaOH.

Two ml blood was extracted through Sep-pak® C_{18} cartridge and then hydrolyzed with NaOH. Bile acids from both bile and serum were treated as hexafluoroisopropyl ester trifluoroacetyl derivatives and measured quantitatively by a Shimadzu GC-9A gas-chromatograph equipped with electron capture detector[2]. A glass column packed 1.5% QF-1/ Chromsorb W AW DMCS (2.1 m × 3.2 mm ID) was used.The operating procedure was as follows: injection and detector temperature, 300℃; column temperature, 240℃; and nitrogen flow-rate 60 ml/min.

Biliary cholesterol saturation index (CSI)

Biliary cholesterol was measured by enzymatic method and phospholipids by Bartlett's method. The total bile acids (TBA) was summed by DCA, CDCA and cholic acid (CA). CSI was calculated according to the critical table of Carey.

Statistical analysis

The results were expressed as $\bar{x} \pm s_{\bar{x}}$. Statistical analysis included both *t* test and correlation test. *P*-values less than 0.05 were considered as significant.

RESULTS

Characteristics of bile acids in serum of gallstone patients

Patients with cholesterol gallstones had significantly higher serum DCA than controls. However, there was no significant difference between the two groups in the other serum bile acids and total bile acids (Table 1). When analyzing the mole percent of serum bile acids (Table 2), we found that DCA was increased in patients with gallstones compared with controls, CDCA was decreased and the ratio of DCA/CDCA increased nearly one-fold.

Correlation between bile acids and CSI

Biliary bile acids were mainly composed of CA, CDCA and DCA with a small fraction of lithocholic acid (LCA). Correlation analysis of 90 samples of gallbladder bile acids showed that DCA and LCA were positively correlated with CSI whereas CDCA was negatively correlated with CSI. No correlation was found between CSI and CA (Table 3). These showed each bile acid had its own characteristics, DCA and LCA were lithogenic whereas CDCA could decrease CSI.

Correlation between biliary and serum bile acids

The concentration of biliary bile acids was much higher than those in serum (Table 4). Significant correlation existed between the mole percent of biliary and serum bile acids, which suggested that serum bile acids concentration might reflect the relative concentration of bile acids in the gallbladder (Table 5).

Table 1 Changes of serum bile acids in the two groups (μmol/L, $\bar{x} \pm s_{\bar{x}}$)

Bile acids	Group with calculi (n = 151)	Control group (n = 256)
DCA*	0.955 ± 0.078	0.696 ± 0.047
CDCA	2.535 ± 0.304	2.084 ± 0.141
CA	1.202 ± 0.134	1.211 ± 0.147
TBA	4.692 ± 0.432	3.290 ± 0.267

*P = 0.002.

Table 2 Mole percent of serum bile acids in the two groups (%, $\bar{x} \pm s_{\bar{x}}$)

Bile acids	Group with calculi (n = 151)	Control group (n = 256)	P
DCA	28.12 ± 1667	23.03 ± 1.04	0.007
CDCA	45.11 ± 2.01	52.40 ± 1.32	0.001
CA	27.51 ± 1.93	24.77 ± 1.09	>0.05
DCA / CDCA	1.76 ± 0.30	0.92 ± 0.14	0.004

Table 3 Correlation between bile acids and biliary CSI (n = 90)

Bile acids	Correlation coefficient	P
LCA	0.288	0.006
DCA	0.311	0.003
CDCA	-0.269	0.01
CA	-0.101	>0.05

Table 4 Biliary and serum bile acids (n = 90, $\bar{x} \pm s_{\bar{x}}$)

Bile acids	Bile (mmol/L)	Serum (μmol/L)	Correlation coefficient*
LCA	1.97±0.18		
DCA	24.80±2.12	1.08±0.11	0.12
CDCA	76.93±4.01	2.35±0.43	0.07
CA	40.27±3.08	1.31±0.19	-0.09
TBA	143.97±7.33	4.65±0.62	-0.07

*P>0.05.

Table 5 Correlation between mole percent of biliary and serum bile acids (n = 90, $\bar{x} \pm s_{\bar{x}}$)

Bile acids	Bile(mole %)	Serum (mole %)	Correlation coefficient	P
LCA	1.89±0.24			
DCA	17.52±1.06	30.15±2.59	0.445	<0.001
CDCA	54.16±1.12	36.66±2.91	0.434	<0.001
CA	26.43±0.96	33.19±3.04	0.313	0.003

DISCUSSION

Biliary cholesterol supersaturation is a prerequisite for the formation of cholesterol gallstones. A positive correlation was found between serum DCA and CSI in bile of patients with stone[3]. Therefore it might be possible to predict biliary CSI just by analyzing serum bile acids, but this study was primarily based on inpatients with gallstone. The present study was performed among normal

population, asymptomatic gallstone patients and healthy individuals, rather than inpatients[3,4]. Among the 407 samples, the mole percent of biliary DCA was found positively correlated with CSI, and serum DCA was increased by about 37% in patients with cholesterol gallstones as compared with control, and the ratio of DCA/CDCA increased nearly one-fold. Hence, there is not only possibility of prediction of CSI in bile, but also characteristic change of DCA in the cholesterol gallstone patients.

Normally, bile acids can be classified into primary bile acids, CA and CDCA, and secondary bile acid, DCA. CA is dehydrogenated to DCA by intestinal bacteria and their secretion is coupled with cholesterol. Bile acid can increase cholesterol secretion and is related to its different hydrophobicity. DCA is more hydrophobic and is followed by greater output of biliary cholesterol than other more hydrophilic bile acids, as CA, CDCA and UDCA. It had been demonstrated by DiDonato *et al*[5] that biliary DCA increased from 5.3% to 43.9% and bile CSI increased simultaneously from 0.9 to 1.34 after given a dose of 3 mg/kg of DCA orally for three to four weeks. One week after its discontinuance, the biliary CSI returned to the original level. DCA could also promote cholesterol crystal formation. The in vitro experiment showed that the nucleation time shortened when the amount of taurodeoxycholic acid increased[1]. Therefore, DCA had a dual effect on supersaturation and nucleation, which contributed to gallstone formation. It was further confirmed by Berr *et al*[6] that increase in hydrophobic bile acids and decrease in its counterparts were the main metabolic disturbance for cholesterol gallstone disease.

Hepatocytes uptake most of the bile acids in the portal vein and then transport them into enterohepatic circulation. The remaining minor portion of bile acids escaped into the systemic circulation are the origin of bile acids in peripheral circulation. They are proportionally related to the composition of bile acid pool[7]. Whiting *et al* suggested that serum bile acids could reflect biliary bile acids, which was confirmed by the present study. As serum DCA is relevant to the relative concentration of bile DCA, the determination of DCA may be useful for the prediction and guiding preventive treatment of gallstone disease[8-10].

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Review

Current status of gene therapy in gastroenterology

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Subject headings gastroenterology; gene therapy; gene transfer; gene expression; DNA, antisense; genes, surpressor, tumor; oncogenes; neoplasms

INTRODUCTION

The potential role of genetic intervention extends from diseases caused by single gene defects, through severe viral infections, to polygenic disorders, such as diabetes mellitus and arteriosclerosis. However, gene therapy can be defined as the introduction and expression of an exogenous gene in human cells for therapeutic benefit, and is conventionally restricted to human diseases associated with single gene defects. There are wider opportunities for genetic intervention and these include strategies to reduce or block gene expression as well as the introduction of nonmammalian genes. The rapid progress in our understanding of some of the molecular mechanisms involved in the pathogenesis of cancer and metabolic disorders, coupled with the development of gene delivery vector technology, has urged us to consider novel genetic approaches to digestive diseases.

There is no shortage of ideas and applications for genetic intervention in human diseases, but there are great limitations not only with the efficiency and targeting of the present generation of gene transfer vectors but also with our incomplete understanding of transcription control.

GENE TRANSFER TECHNOLOGY

The gene delivery technology is advancing rapidly and there have been specific developments that could be translated into gene based therapies for gastroenterological diseases. For example, *ex vivo* transfer methods are being studied extensively using hepatocytes obtained through liver biopsy, partial hepatectomy, and from specimens harvested for liver transplantation. Adult liver cells transiently undergo active proliferation permitting *in vitro* gene transfer even with vectors that require active cell division for entry and expression. Gene transfer may then be facilitated through a number of methods, including viruses, liposome, calcium phosphate coprecipitation, particle bombardment, naked DNA injection, and electroporation. The transfected cells are reintroduced into the host by using, for example, a microcarrier system into the peritoneum, gel beads, hepatocyte coated cell support matrix implanted next to liver tissue, or into the spleen or portal circulation through direct injection.

The spectrum of delivery systems for *ex vivo* gene transfer is broadly applied also to the *in vivo* model. Although the transfer efficiency of liposomes is low, these lipids can be made comparatively easily to high chemical purity and have low immunogenicity, which may permit repeated administrations. They have been used successfully in an *in vivo* model, by topical administration to epithelial cells both in the airways and the intestinal tract and also by the intravascular route. A recent study showed high efficiency transfer of the APC tumor suppresser gene in liposome complexes delivered to normal mouse colonic epithelium by rectal catheter infusion. Almost 100% of epithelial cells expressed the gene for up to four days, which is consistent with the known rate of turnover of this tissue[1]. Intravenous injection of a rat insulin gene expression vector in liposome complexes results in uptake primarily by the liver and spleen. Improvement in hepatocyte uptake can be achieved by incorporating lactosyl ceramide into the phospholipid bilayer; this galactosyl terminal asialoganglioside is specifically recognized by a receptor highly selective for hepatocytes. Many different lipid agents are now being explored for efficacy of DNA transfer and it seems likely that the composition of the complex will have to be optimized for different targets and different routes of administration.

Of the available methods of gene delivery, viruses have been proved the most efficient so far. Achieving viral gene transfer to specific organs for clinical application will be difficult, however, particularly as viral titres 10 to 1000 times higher than those usually attained (typically 10^6 infectious units per milliliter) will be necessary for *in vivo* strategies. There is now extensive experience with retroviruses whose main advantages include their small size and easy manipulation, and with stable colinear integration with host genome. They are comparatively non-toxic and are efficient for gene transfer. Retroviruses persist in up to 5% of hepatocytes three months after injection of an infected hepatocyte cell suspension into the portal vein after partial hepatectomy. The small intestinal epithelium is an attractive target for gene therapy because of its large surface area, easy accessibility, and the presence of stem cells with known locations. Although few studies have yet targeted the intestinal system *in vivo*, marker genes have been transferred to the epithelial surface with retroviral vectors in animal models. Clearly, unless the therapeutic or marker gene is transferred to the stem cells, the rapid turn-over of this specialized

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epithelium would seriously limit potential benefits of delivered genes. Retroviruses have a number of disadvantages, notably the requirement for cells that are actively dividing to permit viral DNA integration, the ability to carry only small DNA sequences, and a small but finite risk of causing insertional mutagenesis as a result of random integration.

Currently alternative viral vectors with potential advantages over retroviruses in specific applications are under development. Adenoviruses can infect non-dividing cells, can be concentrated to high titres, and are comparatively highly efficient vectors. Adeno-associated viruses are ubiquitous and non-pathogenic in humans and can also infect non-replicating cells, but, like retroviruses and adenoviruses, are limited in the size of the foreign gene that can be inserted. This last problem may be overcome by the use of herpes simplex group viruses and possibly even vectors based on hepatitis B virus, which has potential additional advantages of hepatotropism and an ability to integrate with host genome *in vivo*.

GENE THERAPY IN GASTROENTEROLOGY

Strategies for genetic intervention can be divided into five main aspects: replacement or augmentation of gene expression; reduction of expression of genes by antisense or ribozyme technology; genetic prodrug activation; augmentation of immune responses; and polynucleotide vaccination.

Some strategies can be achieved by *ex vivo* gene transfer into isolated human cells, which can then be reimplanted into the host, while others require delivery and expression of genes to target cells *in vivo*—a major challenge with current vector technology. In this review we consider important clinical applications within these categories and outline the directions of study that should lead to clinical trials in the near future.

Cystic fibrosis

Replacement strategies for disorders resulting from a single gene defect are attractive candidates for gene therapy. Inheritance of two mutated copies of the cystic fibrosis transmembrane conductance regulator (CFRT) gene on chromosome 7q22 causes this common autosomal recessive disorder[2]. The CFRT functions primarily as a cyclic adenosine monophosphate activated chloride channel in epithelial cell. The CFRT gene has a complex mechanism of regulation and is expressed mainly in particular classes of epithelial cell. In humans these sites include certain cells of the lung, crypt cell in the ileum, duodenum and colon, the pancreatic ducts, and gall bladder. There is a recent evidence of endogenous CFRT gene expression in intrahepatic biliary epithelial cells, which is consistent with clinical findings of cystic fibrosis induced biliary damage.

Despite recent molecular genetic information regarding the CFRT gene and its product, the exact cause of the mucosal abnormalities in cystic fibrosis is unclear. The changes are not fully explained simply on the basis of loss or poor functioning of a small cAMP activated chloride conductance in the apical membranes of certain specialized epithelial cells. It is not clear how different mutations in the CFRT gene actually cause specific phenotypic presentations of cystic fibrosis.

Despite this problem, substantial progress has been made with gene therapy strategies for cystic fibrosis. The four transgenic mouse models available for study have severe intestinal disease with relative sparing of lung and pancreatic epithelia (unlike those in humans). Correction of the lethal intestinal defect has been shown by transfection of human CFRT by liposomal delivery in a vector under the control of rat intestinal fatty acid binding protein gene promoter. Treated mice survived for up to seven months, well beyond the expected four weeks of the control group, and also showed functional correction of ileal goblet cell and crypt cell hyperplasia and cAMP-chloride secretion. Gene therapy aimed at correcting lung abnormalities in human subjects has entailed the direct introduction of non-mutant CFRT cDNA into the epithelial cells of the respiratory tract in the hope that this will result in sufficient CFRT protein being made in these cells to correct the disease process. Vectors used so far include liposomes and adenoviruses, each has its own drawbacks: the liposomal route is proving relatively inefficient while immunological responses to adenovirus may limitits efficacy. Nevertheless initial data from two phase I clinical trials of cationic liposomal mediated CFRT delivery of a plasmid containing the CFRT cDNA to the nasal epithelium has resulted in over 20% correction of conductance abnormalities in nasal mucosal biopsies and the overall treatment was well tolerated. Attempts have also been made to transfer the CFRT gene into the biliary epithelium *in vivo* using adenoviruses. In one study, injection of the vector directly into the common bile duct during laparotomy resulted in gene expression in nearly all intrahepatic bile duct epithelial cells. Gene expression remained stable after 21 days in epithelial cells of small ducts. These results are encouraging particularly because gene delivery might be achieved by endoscopic retrograde cholangiopancreatography in the future[2].

α_1 antitrypsin deficiency

Approximately 1% of the British population carry the PiZ defect caused by a point mutation in the α_1 antitrypsin gene in chromosome 14, leading to low serum concentrations of α_1 antitrypsin and predisposition to emphysema and liver cirrhosis. Savransky *et al*[3] have proposed one approach to correcting this gene defect in human PiZ GM2522 fibroblasts using the technique of targeted homologous recombination to the gene locus, replacing exon V of the abnormal gene with the exon V counterpart of a normal complementary DNA. Other groups have shown a similar effect in hepatoma cell lines and hepatocytes in rat liver *in vivo* using soluble carrier systems, which utilize the asialoglycoprotein receptors on hepatocytes. It is conceivable that in due course harvested human fetal

hepatocytes identified as possessing the PiZ defect could be subjected to targeted homologous recombination *in vitro* and then reinfused into the portal vein.

Low density lipoprotein (LDL) receptor deficiency

Homozygous LDL receptor deficiency leads to familial hypercholesterolaemia, in which patients have six-to eight-fold increases in plasma LDL-cholesterol values associated with premature atherosclerotic disease and early death. This is a common disease resulting from a mutation in the genitics of the LDL receptors affecting one in 500 of the population. LDL receptors are found in most tissues but it is hepatic expression of the receptor that is most crucial in cholesterol homeostasis. This condition may respond to drug treatment in many cases but transfer of LDL receptor genes to the liver may be a treatment option for a few subjects with intractable hypercholesterolaemia for whom the only treatment option is liver transplantation. Animal studies using the model for homozygous familial hypercholesterolaemia, the Watanabe heritable hyperlipidemic rabbit, have shown successful *ex vivo* retroviral transfer of the receptor gene to 20% of cultured hepatocytes. Transduced cells then expressed receptor levels four or five times higher than normal hepatocyte controls, and after reinjection into the host liver, resulted in lowering total cholesterol value by 50%-70% of its pretreatment value, an effect that lasted over four months. Furthermore there are indications that clinical benefit may result from even partial correction of the total receptor defect, and clinical study has already been underway in the United States using retroviral *ex vivo* gene transfer.

Antisense DNA oligomer treatment: suppressing HBV expression

Using asialo-orosmucoid coupled with poly-L-lysine, both single and double stranded DNA has been delivered specifically to hepatocytes by targeting their asialoglycoprotein receptors. An antisense oligodeoxynucleotide complementary to the polyadenylation signal for human hepatitis B virus was introduced into HepG2 hepatoma cell line by Wu *et al*[4]. This cell line has been transfected with a complete human hepatitis B virus genome and secreted infectious viral into the culture medium. At 24 hours and seven days after exposure to the antisense sequence, HBV DNA values were 80% and 95% lower than controls respectively. Furthermore, by using similar carrier systems, two separate antisense sequences injected into Beijing ducks (an animal model for HBV infection) resulted in suppression of viral replication by over 90% compared with controls.

Replacing defective tumor suppressor genes

In cell culture, malignant properties can often be reversed by inserting normaltumor suppresser genes. The difficulty for corrective strategies lies in delivering actively expressed vectors to each single tumor cell of an established and possibly disseminated cancer *in vivo*. The same argument may not apply for prophylactic therapy to replace gene function in cells of patients with inherited abnormalities of tumor suppresser genes, where it may not be necessary to correct the constitution of every cell to significantly reduce the risk of cancer development within the lifetime of a person.

Normal (wild type) *p53* is involved in the control of cell cycle progression as well as in arresting replication to permit repair in DNA-damaged cells. It may also be involved in restricting precursor po-pulations by mediating apoptosis or programmed cell death. Abnormal or mutant *p53* permits the accumulation of gene mutations and chromosomal rearrangements and has been associated with virtually every sporadic malignancy including gastrointestinal tumors and hepatocellular carcinoma. There is experimental evidence showing the benefits of correcting *p53* abnormalities. Replacement of wild type *p53* using retroviral expression vectors in both human lung cancer cell lines with mouse model of orthotopic human lung cancer resulted in suppression of the malignant phenotype. Furthermore, there was also evidence that the combination of restoration of *p53* function and sequential administration of the cytotoxic drug cisplatin was synergistic in reducing the malignant behavior of these cell lines[5]; this is clearly an important finding that may influence approaches to adjuvant treatment for cancer.

There could also be a role of replacement therapy for *p53* in patients with Barrett esophagus, a condition that may respond poorly to medical treatment. Adenocarcinoma arising in Barrett esophagus is often preceded by mucosal dysplasia. Several researchers have found an association between *p53* mutation and adenocarcinoma related to Barrett esophagus[6]. In addition, recent reports suggest that *p53* dysfunction may participate in the progression from dysplasia to carcinoma, and that there is a correlation between presence of mutant *p53* and increasing dysplastic features[7]. For those patients with histological evidence of progressive dysplasia who are unfit for surgery and refractory to treatment with acid suppression, correction by insertion of wild type *p53* using submucosal endoscopic injection of retroviral or adenoviral suspensions may be an alternative treatment. Clearly such a strategy will require considerable improvement in gene delivery systems and targeting mechanisms for the preneoplastic cells, as well as more sophisticated understanding of transcription control to permit appropriate expression.

The identification of the APC gene participating in familial adenomatous polyposis affecting the large and small intestine, and the DNA mismatch repair gene families (hMSH2, hMLH1, hPMS1, and hPMS2) involved in hereditary non-polyposis colorectal cancer syndrome will stimulate further interest in the replacement of tumor suppresser gene function in stem cells in organs at risk of cancer development. However, the lack of knowledge concerning the temporal and special control of expression of these genes will

delay the application of such prophylactic gene therapy.

Antisense DNA oligomer treatment: suppressing oncogene expression

Antisense oligodeoxynucleotides are short (10 - 15 bases) synthetic nucleotide sequences formulated to be complementary to specific DNA or RNA sequences. By the binding of these nucleotides to their targets, the transcription or translation of a single gene can be selectively inhibited by triggering RNase H degradation of the target RNA and interfering with the processing of pre-mRNA. Examples of antisense oligomers with significant *in vitro* antiproliferative activity include those against *c-myb* in colorectal cell lines, *c-myc* in lymphoma lines, and *bcr-abl* in chronic myeloid leukemia blast cells.

The *ras* oncogenes are obvious potential targets for antisense therapeutics as they are implicated in many solid tumors including more than 75% of pancreatic cancers and colorectal cancers. Inhibition of the *ras* signaling pathway modulates critical aspects of *ras* oncogene mediated transformation in whole cells. The resulting phenotypes include reduced anchorage dependent and anchorage independent growth and morphological reversion of the cells. Recently, reduced biological aggressiveness and loss of anchorage independent growth were reported in experiments using homologous recombination to target *k-ras*. Using antisense oligonucleotides to target regions of *k-ras* mRNA there are some antiproliferative effects in human pancreatic cancer cell lines, but there is wide variability of response rates in different cell lines and little evidence of sequence specificity[8]. Moreover, there is no correlation of antiproliferative effect with reduction in amounts of *k-ras* protein expressed and these agents theoretically directing against cellular genes act instead through unpredictable sequence independent mechanisms. Similar findings have been reported for oligonucleotides designed against *c-myc* and *c-myb* sequences[9]. The amplification of *c-myc*, *c-k-ras*, and *c-Ha-ras* oncogenes in a series of 124 gastric carcinomas did not reveal any new independent prognostic factor. On the contrary, amplification of *c-erb B-2* had a significant negative impact on overall survival. An examination of the tumors involving the thymidine labeling index highlighted the need to identify the most suitable biologic material for estimating proliferative activity in gastric carcinoma[10].

Inserting drug activating 'suicide' genes

There are many examples of genes preferentially expressed in tumors compared with normal tissues. Genetically directed enzyme prodrug therapy (GDEPT) exploits the differences in gene expression between different cell types to increase the specificity of cell destruction by coupling the promoters of differently expressed genes to prodrug activating enzymes. Examples of useful promoters include carcinoembryonic antigen in colorectal cancer, *erbB2* in breast and pancreatic cancer[11], and prostate specific antigen in prostate; the most widely used prodrug activating enzymes are cytosine deaminase (which converts 5-fluorocytosine to the cytotoxic 5-fluorouracil) and herpes simplex virus thymidine kinase (which converts ganciclovir to toxic phosphorylated derivatives). The system is designed so that significant transcription of the enzyme gene is activated only in tumor cells by linking the enzyme gene to transcription control elements (a 'molecular switch') selective for a particular tumor or tissue type. Significant antitumor effects from conversion of 5-FC to 5-FU have been seen in colorectal cancer cell lines transduced with the cytosine deaminase gene under a constitutive promoter[12]. There was also significant regression of tumor volume even when as little as 2% of the tumor mass contained cytosine-deaminase expressing cells. The last effect was also seen when a CEA-HSVTK construct was used to transduce CEA expressing pancreatic cancer cells, which were then engrafted into several combined immune deficient mice. When ganciclovir was given, only 10% of the cells expressed HSVDK[13]. This important feature of the GDEPT system is probably due to a so-called 'bystander effect'¯attributed to transfer of the activated drug from a cell in which it is produced to others in the vicinity lacking the enzyme. There has been some success in targeting tissue such as the gastrointestinal epithelium and pancreas using the carcinoembryonic antigen gene promoter[12], and also targeting tumor specific transcriptional activation using the α-fetoprotein promoter in hepatocellular carcinoma.

Augmentation of the immune response and cancer vaccines

The promise shown by systemic high dose immunotherapy to treat cancer in murine studies has not been fulfilled in human trials except in a few cases of renal cell carcinoma and melanoma. Indeed the initial dose regimens were extremely toxic. It may be more important to deliver a small dose of cytokine at a specific site rather than high doses systematically, which has led to two approaches to cytokine based gene therapy that augment naturally produced immune response to malignancies. The first approach entails the insertion of cytokine genes into cultured tumor infiltrating lymphocytes *ex vivo*. This subset of T cells is critical for the prevention and elimination of tumors, and their antitumor efficacy improves with the transfer of the genes for tumor necrosis factor and interleukin 2. 'Cancer vaccines'¯ constitute the second approach and entail the induction of cytokine expression in the tumor cells so that T cell recognition of tumor antigens is enhanced. Immunostimulatory cytokine genes are transduced into tumor cells *ex vivo*, the tumor cells are irradiated to eliminate malignant activity and reintroduced into the host. Cytotoxic T cells recognize tumor specific antigens presentred on the surface of these cells. They are induced by the local secretion of the transferred cytokine gene product to expand, target, and destroy cancer cells.

In addition to cytokines, a number of other genes are also capable of inducing an antitumor response including allogeneic HLA (human leucocyte antigen) genes and costimulatory molecules such as the B7 family, B7.1 and B7.2. B7 is expressed mainly on antigen presenting cells and serves as costimulatory signals for T cells, by interacting with its ligands CD28 or CTLA-4.

Cytotoxic T cells depend on antigen present in the context of self class I MHC molecules, whereas T helper lymphocytes require activation by antigen present in the context of self class II MHC molecules. Aberrant expression of MHC is a common feature of gastrointestinal cancers. Class I antigens are frequently lost, while class II expression is often unregulated. Polynucleotide vaccination (in contrast with conventional vaccines consisting of peptides, whole tumor cell lysates) has great therapeutic potential in that delivery of genes that express unique oncoproteins such as *k-ras* or *p53* endogenously within a cell and may result in an MHC class I CD8 + response and proliferative activation of cytotoxic T cells, rather than a less effective class II CD4 + response. This may be a further means of breaking down immunotolerance to tumors, which could lead to the generation of tumor specific responses. Mutated forms of the ras protooncogene, in particular, contain potentially antigenic T cell epitomes specific for the malignant phenotype. Certain k-ras mutations that produce new peptides including consensus binding motifs could cause increased immunogenicity as a direct result of differential MHC class I binding. Carcinoembryonic antigen has been utilized for active immunotherapy.

The finding that individual tumor cells often express much higher levels of this antigen suggested that carcinoembryonic antigen may be a target for immunotherapy, initially using DNA vaccines. Although humoral immune responses were seen, there was no evidence of a more powerful cell-mediated cytotoxicity. Secondly, the injected gene was of human sequence, which will be recognized by the mouse host as a foreign antigen, while the product of a gene from the homologous species may be less immunogenic. Thirdly, the challenge with a small inoculum of tumor cells at the end of a course of vaccination is clearly different from the treatment of a patient with extensive metastatic colorectal cancer. Patients with nm23-H1 allelic deletions are 3 times as likely to develop distant metastases as patients without nm23-H1 deletions (relative risk, 3.89; 95% confidence interval, 1.39, 10.89; $P = 0.01$).

PROSPECT IN THE FUTURE

These fields are under intense investigation and real advances are likely in the next decade. In the short-term investigation, further useful improvements are possible from manipulation of the RNA and DNA viruses with which we are already familiar. Development of high efficiency viral packaging systems and refinement of the purification and concentration processes can be expected to improve the titres of viral vectors to values that could permit gene transfer by systemic administration. Targeting of delivery should be possible by incorporating single chain antibodies to cell surface antigens or ligands for transmembrane receptors into the envelope or penton coat proteins of retroviruses and adenoviruses respectively, and there are encouraging signs that this approach can be successful. In the intermediate term, we expect the arrival of 'designer vector' incorporating the most useful elements from both viral and synthetic systems and these can be varied depending on the particular application. For instance, the inverted terminal repeats of adeno-associated virus that mediate stable chromosomal integration can be combined with a backbone of another vector with a large insert capacity such as herpes virus or a bacteriophage. Integration may be enhanced by packaging a functional recombinase enzyme with the gene expression construct in liposomal complexes targeted to a particular class of cells with an antibody. In the long term, basic research will be made into the structure and organization of mammalian chromosomes, which can carry whole clusters of genes with their natural control elements into cells.

The control of gene transcription is extremely complicated and, even for the most intensely investigated systems such as the globin genes, our understanding is still fragmental. While most protocols presently use strong viral promoters to drive expression of recombinant cDNA copies of therapeutic genes, future work must be directed to defining the genomic elements that enable temporal and spatial control of expression through a lifetime. The identification of locus control regions that can insulate gene clusters from interference by surrounding genetic influences has been an important step, and many investigators are now working to understand how the promoter and enhancer/silencer elements of a gene interact with structures within the nucleus. Advances in this area will require parallel deve-lopments in the sophistication of vector design before they can be transferred into practice.

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Clinical Experience

Management of severe acute pancreatitis

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Severe acute pancreatitis is associated with organ failure and/or local complications as necrosis, abscess and fluid collections and pseudocysts. Shock, gastrointestinal bleeding, renal insufficiency, severe metabolic disturbance are common complications whereas adult respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC) and pancreatic encephalopathy are most serious. The degree of lesions varies in different individuals, therefore treatment should be individualized. In recent years, we managed such patients by combined traditional Chinese and western medicine according to Balthazar and Ranson's CT grading[1] with great success, no death occurred in cases with CT grade E and D, and many serious complications have become preventable. The essentials of this management are depicted below.

Inhibiting pancreatic enzyme secretions and activities and decrease of exudation into pancreatic substance, abdominal cavity and retroperitoneal space

Severe acute pancreatitis is often caused by hemorrhage and necrosis and less commonly by interstitial edema, however, the treatment is similar. The pancreatic fluid is rich in pancreatic lipase, trypsin, chymotrypsin, elastase, phospholipase A_2, RNAase and kallikrein and the exudative fluid in the abdominal cavity contained protease, bradykinin, histamine, complement component, phospholipase A_2 and prostaglandins, which can destroy pancreatic tissues and damage the abdominal organs and tissues. Release of cytokines such as IL-1, TNF, PAF, and TXA_2 ET, can cause serious damages to blood vessels with consequent increase of vascular permeability and loss of plasma.

Octreolide can inhibit pancreatic exocrine secretion and gastrointestinal hormones, reduce intraductal pressure, diminish pancreatic autodigestion, decrease PAF activity and reduce exudation from microvessels. It was administered at dosage of 0.1 mg in 20 ml 25% glucose intravenously and followed by 0.5 mg in 1000 ml glucose in saline for 24 hours continuously for 5 - 7 days. Decocting of Rhubarb mixtures (Bupleuri radix 10 g. White peony 10 g, Scutellaria 10 g, Unripe bitter orange 10 g, Magnola bark 10 g, Refine mirabilite 10 g, Rhubarb 10 g) was used concomitantly twice a day, for most serious cases, once every six hours. Rhubarb stabilizes the lysosomal membrane, inhibits secretion and activities of pancreatic lipase, trypsin, chymotrypsin, elastase, kallikrein-kinin relaxes Oddi's sphincter; promotes colonic peristalsis, contraction and emptying, besides, it has broad antibiotic spectrum. Bupleuri radix , Unripe bitter orange 枳实 and Refined mirabilite enhance the small intestinal propulsive function, white peony inhibits pancreatic amylase, Bupleuri radix and Scutellaria lower the elevated temperature due to necrosis. The overall action of this prescription is to promote the drainage of pancreatic fluid and retroperitoneal exudation along the pathway to pancreatic duct and intestinal tract and pass out from the anus. Rhubarb mixture and octretide potentiate each other in inhibiting the enzymatic activities, the former also alleviates the abdominal distention and pain.

Replenishing the diminished blood volume and correcting the hypoalbuminemia

Loss of plasma into peritoneal cavity and retroperitoneal space due to increased vascular permeability leads to hypotension and even shock, this requires early replenishment of blood volume. We infused 400 ml - 600 ml of plasma instantly and 200 ml everyday afterwards till the general condition became stable. Rhubarb can decrease vascular permeability and arrest the exudative process. In fluid replacement, colloidal and crystalline solution should be given in proper proportion, human serum albumin at a dose of 10 g given everyday and balanced solution is preferred in addition to Ringer's solution, glucose in saline and glucose water with supplements of potassium choride to maintain water electrolyte balance at an optimum level. We give 3000 ml - 4000 ml of fluid every day and keep the urinary output over 1000ml, depending on the moistening degree of the surface of tongue, which indicates the normalization of gastrointestinal function and adequate hydration.

Improving intestinal ileus and restoring absorptive and motility function of gastrointestinal tract

Rhubarb mixture is useful in intestinal ileus patients even when peristaltic sound is diminished or nearly absent. When the ventral surface of tongue become moistened, it indicates that the absorptive and motility function of gastrointestinal tract have been restored, then the velocity and fluid volume infusion should be reduced to avoid overloading of the heart and circulation, particularly in the elderly. We do not advocate the use of atropine or gastric decompression.

Total parenteral nutrition and maintenance of intra and extracellular ionic balance

Rhubarb has inhibitory effect on Na+, K+, -ATPase. ATP comsumption is lowered and body catabolism is kept at a lower level. When general condition is stabilized, early institution of total parenteral nutrition as amino acids mixture and intralipid are imminent sodium, postassium, calcium and magnesium salts should be replenished with sodium potassium phosphate 0.1 mol 200 ml as a loading dose, thereby 100 ml everyday for two days and 100ml once every week to maintain intra and extracellular ionic balance and to correct hypocalcemia concomitantly.

Restoring pancreatic microcir culation and perfusion of vital organs

Pancreatic microcirculatory impairment occurs at the early onset of severe acute pancreatitis with microthrombosis in

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most of the cases, which is synchronous with increment of plasma TXA_2 and TXA_2/PGI_2 ratio. TXA_2 is a potent vasoconstrictor and causes platelet aggregation and constricts the arteriole. Also due to the release of many cytokines and free radicals, the PAF constricts the blood vessels, endothelin lowers the tissue blood flow, free radicals damage the pancreatic tissues, all these augment the ischemic damage on the pancreas. Tetra-methylpyrazine can inhibit TXA_2 synthetase activity, decrease the TXA_2/PGI_2 ratio. PGE_1 canimprove pancreatic microcirculation, inhibit platelet aggregation, decrease TXA_2 synthesis, inhibit release of TNF-α, IL-1, IL-6,phospholipase A_2 and the free radicals liberated by neutrophils and macrophages. Exogenous PGE should be given at 300 μg as a loading dose, and 200 μg in 250 ml glucose solution daily thereafter for 5-7 days which might arrest further ischemic damage to the pancreas and increase the perfusion of other organs such as kidney, lung and brain. To achieve the optimum effect, whether it should be given at the onset of the disease process on admission or given afterwards for tissue repair would require further cumulation of experience.

Prevention and treatment of pancreatic infection

Pancreatic infection develops in 10%-20% of patients with severe acute pancreatitis, especially in those with mutiple organal failure and immunocompromized cases. The organisms frequently isolated from infected necrosis and pancreatic abscess are *E.Coli*, *Klebsiella*, *Enterobacter*, *Enterococcus* and other streptococci, occasionally staphylococcus, pseudomonas, anaerobes or fungus[3]. Sources of bacteria are mostly from biliary, urinary, respiratory tract or colon, which are mostly hematogenous. Bacteremia often comes from venous or urinary catheterization, so venous catheter placement should not be over one month and urinary catheter should be avoided. On selection of antibiotics, one should choose relatively liposoluble acidic drugs with high penetrability into the necrotic tissues which have a larger distribution volume and stabilized ionization rate in basic pH. Quinolones and metroindazole can pass through blood-pancreatic barrier and by cell to cell communication or via paracellular pathway or via damaged ducts, and enter the hemorrhagic necrotizing tissues. The antibiotic level is low in ischemic tissues, because the quinolones cannot diffuse back into plasma, its concentration is relatively high in pancreatic tissues, repeated administrations maintain high tissue level and enhance the degree of penetration without serum accumulation. Imipenum penetrates into pancreatic fluid, its level is higher in necrotic fluid than in pancreatic tissues[4]. Aminoglycosides have a low penetrating ability. Metronidazole is a weak base and has a low molecular size and shows a very high rate of penetration. Therefore, clinically we frequently use ciprofloxacin or together with metronidazole or imipenum alone.

Prevention and treatment of complications

Omeprazole 20 mg - 40 mg by intravenous infusion should be given at the beginning to prevent stress ulcer bleeding and acidification of duodenum which might further lead to secretion of pancreatic fluid. Release of myocardial supressive factor by the pancreas would produce tackycardia and negative effect on the heart which can be abolished by naloxone 0.4 mg intramuscularly or 0.06 mg/kg in divided doses intravenously. Naloxone can enhance cardiac contractility and cardiac output.

Adult respiratory distress syndrome is one of the most severe complications, it often arises from delayed correction of shock. Via the circulation, the phopholipase A_2 and trypsin disrupt type II alveolar cell with defective production of surfactant, at the same time vacuolation of macrophages affect the defensive clearing mechanism of the lung. Futhermore, the accumulation of netrophils produce large amounts of free radicals, TXA_2, TNF-α, fibrin and its degraded product, inflammatory mediator as leukotrienes, all participate in the damaging effect on the lungs, resulting in increased vascular permeability and exudation, interstitial pulmonary edema with early hypoxemia and late carbon dioxide retention. Once the diagnosis of ARDS is established, bronchoalveolar lavage is carried out, first on one side then the other, instill dexamethazone 40 mg and aspirate at the same time, this procedure can washout the inflammatory mediators, complement component, coagulation-fibrinolytic products as well as vascular amines. Corticosteroid inhibits IL-1, IL-6, TNF-α, PAF and inflammatory mediators. Serum albumin should be kept at the lower limit of normal. With diffuse interstitial lung edema, PEEP should be used. Early institution of PGE1 and cytosine diphosphocholine 30 mg/kg in glucose water can inhibit phospholipase A_2 and prevent ARDS.

DIC is another serious complication, small dose of heparin in the dosage 75 mg/d - 100 mg/d in divided doses can be given every six hours, anti-thrombin III concentrates 3000 μ/60 ml can be given in half an hour, then 1000 μ every six hours[5]. FDP and D-dimer would decrease, platelet count and fibrinogen increase and bleeding usually ceases.

Obstructive jaundice is usually due to edema of pancreatic head. It will resolve after subsidence of inflammatory edema, only when complicated with common duct stone and deep jaundice, then surgical drainage would be necessary.

Pancreatic encephalopathy is rare, early and energetic treatment at the start?can prevent its occurrence.

Due to the damage of pancreatic β cells, insulin supplements should be given. The hypertriglyceridemia and hyperlactic acid dehydrogenase would resolve after recovery.

All twelve cases were managed medically, only one case with subhepatic abscess larger than 3 cm was aspirated by CT guidance. All of these recovered uneventfully in 1 and a half to 2 months without serious complications.

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Plasma leucine enkephalin content in patients with "Liver-blood deficiency" syndrome and clinical significance *

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Subject headings "Liver-blood deficiency" syndrome/ pathophysiology; enkephalin/blood; radioimmunoassay

INTRODUCTION

Recent studies revealed that the leucine enkephalin (L-EK) was distributed in rat brains with a higher concentration in hypothalamus and little in the pituitary gland[1], but the study on the relationship of L-EK with "liver-blood deficiency" syndrome (LBDS) was lacking. We determined the plasma L-EK levels in LBDS patients with clinical features of iron deficiency anemie (IDA) and/or chronic aplastic anemia (CAA), so as to investigate their relationship.

MATERIALS AND METHODS

Patients and controls

The LBDS was determined by the traditional Chinese medicine (TCM) and the disease entities determined by the modern medicine; an integrated method was used in selecting the objects for study. All patients came from the departments of hematology and integrated TCM and modern medicine of our hospital, most of them were inpatients, and the diagnosis of LBDS was established by two clinicians. Among the 26 patients with LBDS, 23 were iron deficiency anemia (IDA) and 3 were chronic aplastic anemia(CAA), including 7 males and 19 females; age averaged 39.6 ± 9.0 (21 - 66) years. The 30 healthy controls were employees and blood donors of our hospital, including 15 of each sex, and age averaged 32.8 ± 10.2 (21-46) years.

Diagnostic criteria

The diagnostic criteria for IDA was in conformity with the "Diagnostic criteria and curative improvement standards of clinical diseases"[2], and those of CAA accorded with the 1987 Baoji Conference revised standard[3]. The diagnostic standard of LBDS followed the certified standard of our institute[4], including: a. dizziness; b. decreased visual acuity and/or blurred vision; c. numbness of the extremities; d. face, lip and nails pale and malnourishment; e. tongue pale and pulse taut and thready or thready. Patients presenting symptoms of b or c with additional two symptoms and excluding those displaying Yinxu (deficiency of *yin*), Yangxu (deficiency of *yang*) and Qixu (deficiency of *qi*) were diagnosed as LBDS.

Determination of plasma L-EK

Radioimmunoassay was used to determine the plasma level of L-EK. Three ml fasting blood samples were collected at 6-8 a.m. in test tubes containing 50 μl proinin and 40μl EDTA, mixed and centrifuged at 3000rpm for 15min immediately to isolate the plasma and stored at a -20℃ for determination. The reagent kit was provided by the Department of Neurobiology of Second Military Medical University, Shanghai, and the test was performed according to the manual of the kit. The instrument was the FJ-2107PY immuno-automatic counter of Xi'an 262 Factory.

Statistical analysis

The results were expressed as x ± s, and the difference was examined by Student's *t* test.

RESULTS

In the 26 LBDS patients L-EK was 60.83 ng/L ± 21.44 ng/L as compared with 43.22 ng/L ± 17.99 ng/L in 30 healthy controls ($P<0.01$).

DISCUSSION

The opioid peptides, or the so-called endogenous opioid substances include enkephalin, endorphine and dynorphin, the three major categories, with algether about twenty members in this family. Enkephalins come from prepro-enkephalin A, which is composed of 267 amino acids, including six met-enkephalin and one Leu-EK molecule, which was the first isolated opioid peptide in 1975 and the most abundant opioid in the brain. In the cardiovascular system enkephalin is distributed in the atrial and ventricular conductive system and in the nerve fibers of peripheral vascular walls[5,6]. EK is a kind of neural transmitter, which plays an important role in physiologic function[6]; it affects the hypothalamus, pituitary axis functional activities significantly, including the release of luteinizing hormone (LH), the follicular stimulating hormone (FSH) and the thyroid stimulating hormone (TSH)[7]. The functions of FSH and LH on the ovary are to stimulate the production of gametes and ovarian sex hormones. In circumstances of insufficient secretion of FSH and LH, ovarian functional activities are depressed and the secretion of estrogen and progesterone is defective and ultimately results in oligomenorrhea or amenorrhea and anovulation[8]. The result of our study demonstrated a significantly increased plasma level of L-EK in patients with LBDS, and the difference from the healthy controls was very significant. It is suggested that the underlying pathophysiologic basis of oligo or amenorrhea and infertility of female patients with LBDS might be ascribed to the deficient ovarian functional activity partially induced by the obviously increased enkephalin as shown by the increased plasma level of L-EK in our study.

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