Editorial

Association between *Helicobacter pylori* and gastric cancer: current knowledge and future research

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Subject headings stomach neoplasms/etiology; stomach neoplasms/epidemiology; stomach neoplasms/ prevention and control;DNA;mutation; Helicobacter pylori;helicobacter infection

In 1994 Helicobacter pylori infection was classified as a definite (class 1) carcinogen by the International Agency for Research on Cancer (IARC) on the basis of available epidemiological and clinical research[1]. However, data collected over the past 3 years has failed to provide unequivocal support for the conclusion. The mechanisms by which H. pylori increases the risk of developing gastric cancer remain unclear, despite many significant experimental observations.

EPIDEMIOLOGICAL STUDIES

Association between incidence of gastric cancer and prevalence of H. pylori infection.

While gastric cancer is the second most common cause of cancer death, the incidence has decreased dramatically in some developed countries over the past decades[2,3]. There is also good evidence that the prevalence of *H. pylori* infection has dropped markedly in these countries, and it is unlikely that these two events have happened coincidently. Recent studies has shown that the geographic variation in the incidence of gastric cancer is related to the prevalence of *H. pylori* infection; an increased incidence and mortality of gastric cancer in a region is strongly associated with a high prevalence of *H. pylori* infection in the population within that region[4]. However, lack of association between *H. pylori* and gastric cancer has been reported in some areas of the world, such as the Mediterranean and Africa where the prevalence of *H. pylori* is high but the incidence of gastric cancer is low[5].

Case control and prospective studies

Early case control studies provided convincing support for the association between *H. pylori* infection and gastric cancer[5,7]. In 1994, Forman summarized all available case control studies and concluded that the relative risk for development of gastric cancer was 3.8 fold higher in patients with *H. pylori* infection than those without[6]. However, more recent epidemiological studies have shown inconsistent results[5,8-13]. Two studies carried out in Chinese populations showed nosignificant association between *H. pylori* infection and gastric cancer[8,9], and this finding has been replicated in recent Japanese and Korean studies[11-13].

One limitation of current epidemiological studies is that most studies did not adjust the potential confounding factors such as dietary or socioeconomic characteristics, thus bias may exist[5]. For example, poor living conditions are usually related to a high prevalence of *H. pylori* infection and a high incidence of gastric cancer. Misclassification is another limitation in current epidemiological studies. It is widely accepted that *H. pylori* infection is lost with advancing preneoplasia (such as atrophy, intestinal metaplasia and dysplasia) and that serum titres drop correspondingly[14,15]. Cases are more likely to have advanced preneoplasia than the controls, so that *H. pylori* infection may have disappeared in a substantial proportion of cases. These cases may be classified as *H. pylori* negative since the infection is usually assessed at the time of diagnosis of gastric cancer. Misclassification may also result from the application of serological assays with low sensitivity and specificity for *H. pylori* detection, and from misdiagnosis of gastric cancer. This significantly influences the outcomes of analysis, resulting in underestimation of relative risks[14]. When the misclassification errors are taken into account, it is rare to find studies that do not link *H. pylori* infection to gastric cancer. Therefore, understanding the limitations of these

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epidemiological studies is critical in interpreting the research findings.

EXPERIMENTAL OBSERVATIONS

Cytotoxic strains

Approximately 50% of *H. pylori* strains express the CagA protein. It has been shown that persons with CagA-expressing strains are more likely to develop atrophic gastritis, intestinal metaplasia and gastric cancer than those infected with CagA negative strains[16,17]. Thus, variation in CagA prevalence among populations may explain some of the discrepancies observed in the case-control studies. In populations with a high prevalence of CagA positive strains, *H. pylori* infection is more likely to beidentified as a cancer risk factor. For example, the prevalence of CagA positive strains is substantially higher in Europeans than in African-born subjects[17]. Similarly, CagA positive strains are more prevalent in blacks than in whites in the USA[17]. However, this remains controversial as studies from Asian countries, such as China, Japan and South Korea, failed to show that infection with CagA po-sitive strains is associated with these lesions[18-21].

DNA alternations

There is no evidence, so far, that *H. pylori* infection is, in itself, directly genotoxic or mutagenic. Thus, it is unlikely that there is some direct interaction between the bacterium and host DNA which leads to mutations and transformed cell phenotypes. However, *H. pylori* produces a variety of substances that may indirectly cause DNA damage of the gastric epithelial cells[4]. These products include ammonia or ammonium-containing chemicals, phospholipases, and cytotoxin(s). All these factors may impair the host defence and render the gastric epithelial cells prone to the activity of direct-acting carcinogens.

The host's immune response to *H. pylori* may eventually result in DNA damage. In theory, stimulation of leukocyte cells by a bacterial infection should lead to the activation of both specific and non specific immune defence mechanisms that would cause limitation and resolution of the infection. However, the human immune response to *H. pylori* is not capable of eradicating the infection from the stomach, as the bacteria can avoid the human immune defence system. Thus, stimulation of the leukocyte cells continues as long as the infection persists. This toxic response may, over an extensive time period, lead to structural and biological damage to the gastric epithelium. For example, the excessive production of reactive oxygen metabolites by stimulated neutrophil polymorphs and monocytes may cause extensive DNA damage and molecular mutation[22]. It has been shown that *H. pylori* infected gastric epithelium has a significantly higher level of these compounds than normal epithelium and that eradication of *H. pylori* infection returned to normal levels[23]. Genetic damage by persistent inflammation may be mediated by other chemicals, such as oxidates of nitrogen, and lead to the formation of N-nitroso-compounds and nitric oxide which can induce DNA damage.

Cell division is vital to the development of cancer and an elevated rate of mitosis increases the likelihood of a somatic DNA mutation. Studies in varied populations have shown that *H. pylori* infection is associated with an approximately doubling of cell turnover and that the turnover level returns to the normal level following eradication of the infection[24,25]. It has also been shown that *H. pylori* increases epithelial cell proliferation *in vitro*[26]. On the other hand, *H. pylori* infection has been reported to induce epithelial apoptosis (programmed cell death) *in vivo*[27-29] and eradication of the infection results in a significant reduction in apoptosis. Moreover, a recent study showed that increased cell proliferation was not associated with a corresponding increase in apoptosis in patients infected with cagA+ strains, indicating that failure to delete cells with genetic damage may result in malignant transformation[30].

Telomeres are specialised structures at the ends of chromosomes that are important in protection and replication of chromosomes. Normally, there is progressive shortening of telomeric repeats with each cell division, whereas germline cells compensate for the end replication problem by telomerase expression which permits the synthesis of telomeres, telomerase activity is not expressed at detectable levels in normal somatic cells. So, telomeres in somatic cells progressively shorten with each cell division. Telomere reduction could cause chromosome instability and additional genetic changes, resulting in increased cell proliferation and reactivation of telomerase. Studies have shown that telomerase activity can be detected in most gastric cancers as well as precancerous lesions such as gastric intestinal metaplasia and adenoma, suggesting that the expression of telomerase activity is an early event of gastric carcinogenesis[31-32]. A recent study from Japan showed that the level of human telomerase RNA (hTR) and telomerase activity increased in parallel with the density of *H. pylori* infection, which was also associated with the grade of intestinal metaplasia[33]. This finding suggests that *H. pylori* infection may be a strong trigger for hTR overexpression in intestinal metaplasia, which may lead to telomerase reactivation.

Microenvironmental biophysiological changes

Ascorbic acid (Vitamin C) is a critical antioxidant

which has important functions as a scavenger of reactive oxygen species and inhibits N nitrosation. This antioxidant property of ascorbic acid plays a central role in the chemoprevention against gastric cancer. It has been shown that *H. pylori* infection is associated with a significant decrease in the concentration of ascorbic acid in gastric juice, and that eradication of the infection normalizes the level of ascorbic acid[34,35]. Similarly, the gastric level of β carotene, which is also an antioxidant, is significantly decreased in patients with *H. pylori* infection[36].

FUTURE RESEARCH

Epidemiology

Current epidemiological studies are limited by their small sample size and failure to adjust potential confounders. Well designed prospective and case control studies using large populations are still needed. These may confirm or refute the established "guilty" sentence for *H. pylori* infection in the development of gastric cancer.

Pathogenesis

The model of pathogenesis of gastric cancer has been proposed as a multi-step process; *H. pylori* infection may progress to acute gastritis, chronic gastritis, atrophic gastritis, then intestinal metaplasia, dysplasia, and eventually gastric cancer[23]. However, the role of *H. pylori* infection in each step of this progression remains unclear. Moreover, the exact "point of noreturn" is unknown. Therefore, investigation of the association of *H. pylori* infection and precancerous lesions may provide key answers to the issues. Recently, it has been reported that *H. pylori* infection is associated with antralization (presence of antral type mucosa) at the gastric incisura, body and fundus, which is, in turn, related to intestinal metaplasia at these sites[37]. Whether antralization is a step before intestinal metaplasia, and is reversible, needs to be clarified.

Preventive strategies

At present, an eradication rate of up to 95% has been achieved with the triple therapy and serological or urea breath tests with high sensitivity and specificity for detection of *H. pylori* infection are available[38]. If *H. pylori* infection is proven to be an initiating factor in the development of gastric cancer, then, screening and eradication of *H. pylori* with antimicrobial therapy in large scale populations, in particular, those at high risk, should be considered. However, feasibility, safety and appropriate timing of this strategy remains to be determined. Moreover, side effects and the potential spread of drug resistant strains limits its practical application.

Encouraging results have been reported on the vaccination against *H. pylori* infection. Studies using animal models have shown that immunization against *H. pylori* not only prevents the infection but also can eradicate it£Û39£Ý. Vaccination as the optimal strategy in humans will be confirmed in the management of *H. pylori* infection.

CONCLUSIONS

Preliminary epidemiological data support the link between *H. pylori* infection and the development of gastric cancer. However, well-designed prospective and case-control studies using large populations are still required. Moreover, the mechanisms through which *H. pylori* infection leads to gastric cancer are not fully understood, and studies on this issue are urgently needed. Further research should also explore the possibility of preventing gastric cancer by eradicating *H. pylori* infection, with vaccines.

REFERENCES

1 International Agency for Research on Cancer, Schistosomes, Liver flukes and *Helicobacter pylori*. IARC monographs on the evaluation of carcinogenic risks to humans, 1994;61:177-241
2 Zheng T, Mayne ST, Holford TR, Boyle P, Liu W, Chen Y et al. The time trend and age period cohort effects on incidence of adenocarcinoma of the stomach in Connecticut from 1955-1989. Cancer, 1993;72:330-340
3 Sipponen P, Kimura K. Intestinal metaplasia, atrophic gastritis and stomach cancer: trends over time. Eur J Gastroenterol Hepatol, 1994;6(Suppl 1):S79-83
4 Xia HX, Forman D, Talley NJ. *Helicobacter pylori* infection and gastric cancer. In: Fan XG, Xia HX, eds. *Helicobacter pylori* infection: basic principles and clinical practice. Changsha: Hunan Science and Technology Press,1997:101-108
5 Palli D. Gastric cancer and *Helicobacter pylori*: a critical evaluation of the epidemiological evidence. Helicobacter, 1997;2(Suppl 1):S50-55
6 Forman D, Webb P, Parsonnet J. *H pylori* and gastric cancer. Lancet, 1994; 343:243-244
7 Talley NJ, Xia HHX. Gastric cancer: an infectious disease? Cur Therapy, 1996; 37:17-20
8 Eslick GD, Talley NJ. *Helicobacter pylori* infection and gastric carcinoma: a meta analysis. Gastroenterology, 1997;114:(in press)
9 Lin JT, Wang LY, Wang JT, Wang TH, Yang CS, Chen CJ. A nested case-control study on the association between *Helicobacter pylori* infection and gastric cancer risk in a cohort of 9775 men in Taiwan. Anticancer Res,1995;15:603-606
10 Webb PM, Yu MC, Forman D, Henderson B, Newell DG, Yuan JM et al. An apparent lack of association between *Helicobacter pylori* infection and risk of gastric cancer in China. Int J Cancer, 1996;67:603-607
11 Watanabe Y, Kurata JH, Mizuno S, Mukai M, Inokuchi H, Miki K et al. *Helicobacter pylori* infection and gastric cancer. A nested case-control study in a rural area of Japan. Dig Dis Sci, 1997;42:1383-1387
1 2 Kimura K, Satoh K. What remaining questions regarding *Helicobacter pylori* and associated diseases should be addressed by future research? View from far east. Gastroenterology, 1997;113:S155-157
13 Kim HY, Cho BD, Chang WK, Kim DJ, Kim YB, Park CK et al. *Helicobacter pylori* infection and the risk of gastric cancer among the Korean population. J Gastroenterol Hepatol, 1997;12:100-103
14 Parsonnet J. Lesions from the epidemiology of *Helicobacter pylori* and cancer. In: Hunt R. Tytgat GNJ. eds. *Helicobacter pylori*: basic mechanisms to clinical cure. Dordrecht: Kluwer Academic Publishers, 1996:238-244
15 Xia HH X,Talley NJ. *Helicobacter pylori* infection, reflux esophagitis and atrophic gastritis: an unexplored triangle. Am J Gastroenterol,1998;93:(in press)
16 Blaser MJ, Perez Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH et al. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res, 1995;55:2111-2115
17 Parsonnet J, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. Gut, 1997;40:297-301
18 Mitchell HM, Hazell SL, Li YY, Hu PJ. Serological response to specific *Helicobacter pylori* antigens: antibody against CagA antigen is not predictive of gastric cancer in a developing country. Am J Gastroenterol, 1996;91: 1785-1788
19 Shimoyama T, Fukuda S, Tanaka M, Mikami T, Saito Y, Munakata A. High prevalence of the cagA-positive *Helicobacter pylori* strains in Japanese asymptomatic patients and gastric cancer patients. Scand J Gastroenterol, 1997;

32:465-468
20 Matsukura N, Onda M, Kato S, Hasegawa H, Okawa KI, Shirakawa T et al. Cytotoxin genes of *Helicobacter pylori* in chronic gastritis, gastroduodenal ulcer and gastric cancer: an age and gender matched case control study. Jpn J Cancer Res, 1997;88:532-526
21 Miehlke S, Kim JG, Small SM, Graham DY, Go MF. Lack of association between the prevalence of the cagA gene in *Helicobacter pylori* and gastric adenocarcinoma. Gut,1995;37(Suppl 1):A77
22 Davies GR, Simmonds NJ, Stevens TRJ, Sheaff MT, Banatvala N, Laurenson IF et al. *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production *in vivo.* Gut, 1994;35:179-185
23 Correa P. Human gastric carcinogenesis: a multistep and multifactorial process: first American cancer society award lecture on cancer epidemiology and prevention. Cancer Res, 1992;52:6735-6740
24 Cahill RJ, Xia H, Kilgallen C, Beattie S, Hamilton H, O'Morain C. Effect of eradication of Helicobacter pylori infection on gastric epithelial cell proliferation. Dig Dis Sci, 1995;40:1627-1631
25 Lynch DAF, Mapstone NP, Clarke AMT, Sobala GM, Jackson P, Morrison L et al. Cell proliferation in *Helicobacter pylori* associated gastritis and the effect of eradication therapy. Gut, 1995;36:346-350
26 Fan XG, Kelleher D, Fan XJ, Xia HX, Keeling PWN. *Helicobacter pylori* increase proliferation of gastric epithelial cells. Gut, 1996;38:19-22.x915
27 Dong QJ,Liu W, Zheng X, Xiao S, Shi Y, Peng Y. Effect of *Helicobacter pylori* on gastric epithelial apoptosis.Chin J Intern Med, 1997;36:751-753(in Chinese)
28 Hahm KB, Lee KJ, Choi SY, Kim JH, Cho SW, Yim H et al. Possibility of chemoprevention by the eradication of *Helicobacter pylori*: oxidative DNA damage and apoptosis in *H. pylori* infection. Am J Gastroenterol,1997;92:1853-1857
29 Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B et al.Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. Cancer Res,1996;56:3238-3243
30 Peek RM, Moss SF, Tham KT, Perez Perez GI, Wang S, Miller GG et al. *Helicobacter pylori* cagA+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. J Natl Cancer Inst, 1997;89:863-868
31 Ahn M J Noh YH, Lee YS, Chung TJ, Kim IS, Choi IY et al. Telomerase activity and its clinicopathological significance in gastric cancer. Eur J Cancer, 1997; 33:1309-1313
32 Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y et al. Telomerase activity in gastric cancer. Cancer Res, 1995;55:3258-3262
33 Kuniyasu H, Domen T, Hamamoto T, Yokozaki H, Yasui W, Tahara H et al. Expression of human telomerase RNA is an early event of stomach carcinogenesis. Jpn J Cancer Res, 1997;88:103-107
34 Sobala GM, Schorah CJ, Shires S, Lynch DA, Gallacher B, Dixon MF et al. Effect of eradication of *Helicobacter pylori* on gastric juice ascorbic acid concentration. Gut, 1993;34:1038-1041
35 Banerjee S, Hawksby C, Miller S, Dahill S, Beattie AD, McColl KE et al. Effect of *Helicobacter pylori* and its eradication on gastric juice ascorbic acid. Gut, 1994;35:317-322
36 Zhang ZW, Patchett SE, Perrett D, Domizio P, Farthing MJG. Gastric mucosal and luminal β carotene concentrations in patients with chronic *H. pylori* infection. Gut, 1996;38(Suppl 1):A3 (abstract)
37 Xia HH X, Kalantar J, Ma Wyatt J, Adams S, Cheung K, Talley NJ. *Helicobacter pylori* associated antralization of proximal gastric mucosa is linked to an increased risk of development of intestinal metaplasia. Gastroenterology, 1998;114:(in press) (abstract)
38 Xia HH X,Talley NJ. Prospects for improved therapy for *Helicobacter pylori* infection. Exp Opin Invest Drugs, 1996;5:959-976
39 Corthesy Theulaz I, Porta N, Glauser M, Saraga E, Vaney A C, Haas R et al. Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against Helicobacter infection in mice. Gastroenterology, 1995;109:115-121

Original Articles

Analysis of N-*ras* gene mutation and p53 gene expression in human hepatocellular carcinomas*

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Subject headings liver neoplasms; carcinoma, hepatocellular; genes, p53; genes, ras; mutation; gene expression; polymerase chain reaction; immunohistochemistry

Abstract

AIM To study the relationship between N-*ras* gene mutation and p53 gene expression in the carcinogen-esis and the development of human hepatocellular carcinomas (HCC).

METHODS The N-ras gene mutation and the p53 gene expression were analyzed in 29 cases of HCC by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and immunohistochemistry.

RESULTS Thirteen cases of HCCs were p53 positive (44.8%), which showed a rather high percentage of p53 gene mutation in Guangxi. The aberrations at N-*ras* codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumorous liver tissues. More than 2 point mutations of N-ras gene were observed in 22 cases (75.86%). Twelve cases (41.37%) of HCCs showed both N-*ras* gene mutation and p53 gene expression.

CONCLUSIONS N-*ras* gene and p53 gene may be involved in the carcinogenesis and the development of HCC. That 38% of HCCs with N-ras gene mutation did not express p53 protein indicates that some other genes or factors may participate in the carcinogenesis and the development of HCC.

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INTRODUCTION

The hepatocellular carcinogenesis is presumably a multiple-step process and is influenced by many factors. The accumulation of genetic changes is necessary for emergence of hepatocellular carcinoma (HCC). Activation of pro-oncogenes and inactivation of tumor suppressor genes might be related to the carcinogenesis and the development of HCC. It has been reported that there was mutation of tumor suppressor gene p53 and overexpression of N-*ras* oncogene in human HCC[1-3], but there have been few reports on the relationship between N-*ras* gene mutation and p53 gene expression. We analyzed the N-*ras* gene mutation by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and p53 protein by immunohistochemistry in 29 cases of human HCC in order to explore the relationship between these two kinds of genetic changes and human HCC.

MATERIALS AND METHODS

Clinical samples

Twenty-nine HCC surgical resected specimens were collected in Guangxi Cancer Institute during the period of 1987-1992, 28 of them had HCC adjacent liver tissues. All samples were fixed with 10% formalin, embedded in paraffin and stained with haematoxylin and eosin (HE).

Immunohistochemistry

Immunostaining was performed using a streptavidin-biotin immunoperoxidase method. p53 protein was detected with monoclonal anti p53 antibody (Oncogene Science, PAb DO1) and Strept ABC kit (DAKO A/S Denmark). The tissues were firstly microwaved 5min, 4 times and then used for immunohistochemistry.

DNA extraction

Genomic DNA was prepared by the proteinase K-Phenol-Chloroform extraction method.

PCR-SSCP

Oligomers that flank codon 2-37 of N-*ras* genes was synthesized as a primer by the Department of Molecular Medicine in King's College Hospital, UK. One of them was 5'-end labelled with r-^{32}P ATP by T4 Polynucleotide Kinase reaction. The

primer sets were: 5'-GACTGAGTACAAACTGGTGG-3', 5'-GGGCCTCACCTCTATGGTG-3'.

The amplified product obtained by the PCR is 118bp. 0.1μg DNA taken from tissues, which was added with 9μl PCR mixture (containing 1pmol/L primer, 0.2mmol/L dATP, dGTP, dCTP, dTTP, 0.25U Taq DNA polymerase, 50mmol/L KCl, 10 mmol/L Tris, 2.5mmol/L MgCl2 and 0.45% Tween 20), covered with mineral oil. PCR reaction underwent 5min denature at 94℃, then went to 35 cycles. One cycle included 30sec denature at 95℃, 1min annealing at 55℃, 1min 30sec extension at 72℃, and finally 10min extension at 72℃.

Two μl PCR product was added to 2μl dilution containing 95% formamide, 20mmol/L EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol dye. The mixture was denatured 5min at 95℃, then applied to a 6% polyacrylamide gel (21cm×40cm ×0.4cm) with 1×TBE buffer at 45mA current and 45 Watt in cold room (4℃) for 4-5 hours. The gel was dried at 80℃ and autoradiographed at -70℃.

RESULTS

Pathologic histology

The tumor specimens were all diagnosed as hepatocellular carcinomas. The histological diagnosis of tumor adjacent liver tissues were: 14 (50%) cases of chronic hepatitis, including chronic active type (CAH) and chronic persistant type (CPH); 6 of them had liver cirrhosis with CAH, 6 had liver cirrhosis and 2 cases acute hepatitis.

Point mutation of N ras gene

The N-*ras* PCR-SSCP showed only 3 bands in normal control samples including human liver tissues (2 cases) and human placenta DNA (1 case). These 3 normal bands were also clearly discerned in all HCC and their adjacent tissues. Some of detected specimens presented mobility shift bands, which was the point mutation in N-ras gene. The aberrations at N-*ras* codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumor liver tissues, respectively. There was no significant difference between them (χ^2 test, $P>0.05$). Twenty-two of 29 HCC (75.86%) showed 2-5 mobility shifts.

p53 protein detection

p53 protein was mainly localized in HCC cell nucleus. Both nuclei and cytoplasmic staining were observed in 75% of p53 protein positive HCC. Non-tumor liver tissues showed only cytoplasmic type of p53 protein. p53 protein positive rate was 44.8% (13/29), 12 (41.37%) of them had N-*ras* mutation as well.

DISCUSSION

PCR-SSCP analysis is based on single strand DNAs of the same nucleotide length in which the nucleotide sequences different at only one or some positions can be separated by polyacrylarmide gel electrophoresis. DNA polymorphisms at a variety of positions in a fragment could cause a difference in its conformation and result in change in mobility of the single strands on gel electrophoresis.

In HCC, N-*ras* was first proved as one of the transforming genes[4], which belongs to G protein family. When it is converted to active oncogene by point mutation, chromosome rearrangement or gene amplification, the signal transmission of cell membranes may change, which drives cell division, results in abnormal differentiation and finally forms neoplasm. Enhanced expression of N-*ras* gene in human HCC has been reported[3]. The 'hot spot' mutation at codon 12, 13 or 61 in the N-*ras* gene has been described in many kinds of human cancers[5]. But no point mutation around these 'hot spot' was found in HCC from South Africa[6] and Japan[5]. In China, in FANG Dian Chun's study[7], 37.2% (16/43) of HCC showed N-*ras* gene point mutation at codon 12 by PCR-restriction fragment length polymorphisms (PCR-RFLP). In our study, the PCR product contained codon 12, 13 of N-*ras*. This gene point mutation was found in 79.31% (23/29) of HCC, indicating that the frequency is rather high in HCC from Guangxi region. In contrast to the reports about no N-*ras* gene point mutation or only one mutation at codon 12 in HCC, we found that 75.86% (22/29) of HCC had 2.5 point mutations around codon 2-37. This data showed that the mutation positions were not limited at codon 12 or 13. Therefore, if we only investigate the 'hot spot' mutation of N-*ras* gene, there is a possibility of missing mutations in other regions of the gene. The mobility shifts scattered at different positions. No clustering position could be considered as a hot spot in this study. We do not know the exact codon of mutation in this fragment by PCR-SSCP analysis. They could be confirmed by DNA sequencing.

SSCP bands can be clearly discerned in HCC (79.31%) and their adjacent liver tissues (80.77%). There was no significant difference between them, $P>0.05$. Most of these non-tumorous liver tissues presented as chronic hepatitis and/or liver cirrhosis, which was indicative of a close correlation with HCC. Our result indicates that N-*ras* gene mutations are involved in the carcinogenesis and development of HCC.

Many studies have shown that p53 abnormalities were involved in the genesis of HCC[1-2]. One of the most common mechanisms leading to functional defect of this tumor suppressor gene in carcinogenesis is point mutations and expression of a conformationally altered protein that is immunohistochemically identifiable. Wild-type (normal) p53

protein has a very short half-life[8] and can not be identified immunohistologically. In contrast, most mutant p53 protein becoming more stable with an extended half-life (4-5 hours) and being overexpressed in cells were readily detected by immunohistochemistry. We examined 29 HCC for p53 protein by ABC immunohistochemistry, 13 cases were found positive, accounting for 44.8% of HCC, which showed a high frequency of p53 gene mutation in HCC in Guangxi area.

It has been evidenced that mutation of the tumor suppressor gene p53 can convert *ras* gene into oncogene[9]. The mutant p53 gene cooperation with mutant rasgene in cell transformation has been proved[10]. We noticed that 12/29 (41.37%) cases of HCC had both mutations of N-*ras* gene and p53 gene. The result indicates that the mutations of N-*ras* gene and p53 gene may play an important role in the carcinogenesis and maintenance of HCC. It is interesting that 38% of HCC with N-*ras* mutations was p53 protein negative. The explanation might be that some other genes or factors except N-*ras* and p53 participated in the formation and maintenance of HCC in Guangxi region.

REFERENCES

1 Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. Nature, 1991;350(6317): 429-431
2 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinoma. Nature,1991;350(6317): 427-428
3 Farshid M, Tabor E. Expression of oncogenes and tumor suppressor genes in human hepatocellular carcinoma and hepatoblastoma cell lines. J Med Virol,1992;38(4):235-239
4 Gu JR, Hu LF, Wan DF, Tian PK, Guo C, Huang LH. Study on N-rasgene: the human primary liver cancer transformation gene. Tumour, 1985;5(2): 52-55
5 Tada M, Omata M, Ohto M. Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. Cancer Res, 1990;50(4):1121-1124
6 Leon M, Kew MC. Analysis of ras gene mutations in hepatocellular carcinoma in Southern African blacks. Anticancer Res, 1995;15(3):859-861
7 Fang DC, Luo YH, Lu R, Xiao WH, Liu WW, Men RP et al. Studies of the point mutation of ras oncogene and suppressor gene p53 in 43 cases of hepatocellular carcinoma. Acta Acad Medi Militaris Tertlae, 1994;16(5):340-343
8 Slagle BL, Zhou YZ, Butel JS. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus positive hepatocellular carcinomas.Cancer Res, 1991;51(1):49-54
9 Harris AL. Telling changes of base. Nature, 1991;350(6317):377-378
10 Hinds P,Finlay C,Levine AJ. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol,1989;63(2): 739-746

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Partial isolation and identification of hepatic stimulator substance mRNA extracted from human fetal liver*

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Subject headings fetal liver tissue; RNA, messenger; hepatic stimulator substance; hepatocyte proliferation

Abstract

AIM To partially isolate and identify hepatic stimulator substance mRNA from human fetal liver tissues.

METHODS The poly (A)mRNA was extracted from human fetal liver tissues of 4-5 month gestation, fractionated by size on sucrose gradient centrifugation, translated into protein from each fraction *in vitro* and then its products were tested for HSS activity.

RESULTS Twenty-two 500 μg total RNA was obtained from human fetal liver tissues and pooled. mRNA of 420 μg was yielded, processed by oligo (dT)-cellulose column chromatography, then was size-fractionated by ultracentrifution on a continuous sucrose density gradient (5%-25%), and separated into 18 fractions.Translated products of mRNA in fraction 8 and 9 could produce a two-fold increase in the incorporation of ^{3}H-TdR into DNA of SMMC-7721 hepatoma cells and in a heat resistant and organ-specific way.

CONCLUSION The partially purified HSS mRNA was obtained and this would facilitate the cloning of HSS using expression vectors.

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INTRODUCTION

Besides hormones such as insulin and glucagon, growth factors may be implicated in the complex regulation of a constant liver mass in the adult animal. Transforming growth factor (TGF)-α, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are important stimulators of hepatocyte proliferation, but their activities are not liver specific. On the contrary, hepatic stimulator substance (HSS) is a hepatic specific regulator found in the weanling and regenerating livers from rat, rabbit and pig[1]. We and others[2,3] have demonstrated the presence of stimulator factors in human fetal livers. Its purification and characterization have been reported[4,5]. Furthermore, we also have demonstrated that human HSS is the product of gene expression of fetal liver tissues[6]. But the sequencing of HSS so far has been unsuccessful. To facilitate cDNA cloning of human HSS, we approached the partial characterization of HSS mRNA extracted from human fetal liver tissues, with the technic of fractionation of sucrose gradient centrifugation and translation in vitro.

MATERIALS AND METHODS

Materials

RPMI 1640 was from Gibco Lab, Grand Island, NY; oligo(dT)-cellulose column was from Pharmacia Ltd, Milwaukee, WI; wheat germ translation system; cell titer 96$^{(TM)}$ non-radioactive cell proliferation assay were from Promega Ltd, USA.

Cells lines and culture. The following cells were maintained in monolayer (or suspension) culture in RPMI 1640 medium containing 10% (vol/vol) newborn calf serum: HTC, rat hepatoma cell line; SMMC-7721, human hepatoma cell line; HL-7704, human adult hepatocyte line; HL-60, human promyelocytic leukemic cell line; K562, human erythroid leukemic cell line. Primary tissue cultures (liver, spleen and kidney) were prepared as described[6].

Preparation of poly (A)+mRNA. Total RNA was extracted from human fetal liver tissues of 4-5 month gestation by a modified procedure from Chomczynsky *et al*'s[7]. The extracted RNA was ap-

plied onto an oligo(dT)-cellulose column to obtain purified mRNA.

Sucrose gradient fractionation of poly(A)+mRNA. Poly(A)+mRNA was fractionated on 5%-25% sucrose gradient on SW41 rotor essentially as described by Buell GN *et al*[8]. Briefly, poly(A)+mRNA samples were lyophilized to remove ethanol and dissolved in 0.5 ml of TE buffer, heat-treated at 65℃ for 10min and cooled rapidly in ice. The poly(A)+mRNA of up to 100μg per gradient was layered directly on 5%-25% (w/v) linear sucrose gradients made in TE buffer and centrifuged in an SW41 rotor at 41 000 rpm for 15 h at 20℃. Eighteen fractions (each 1ml) were collected and mRNA in each fraction was precipitated by ethanol.

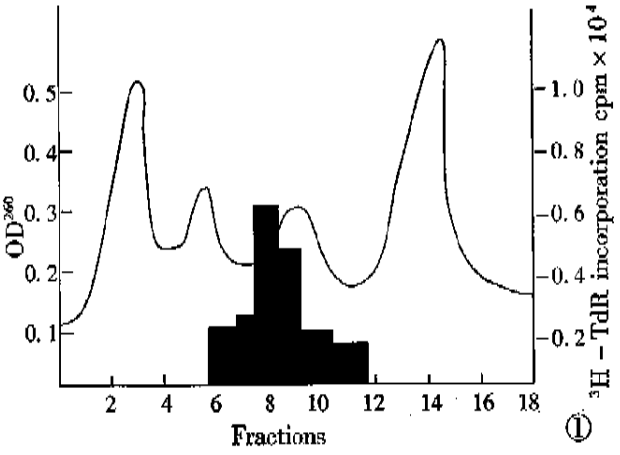
Translation in wheat germ system. mRNA 10 mg/L was heated at 67℃ for 10min, immediately cooled on ice and added to the cell-free protein synthesis system (Promega LTD) according to the manufacturer's instructions. Then the wheat germ extract components were heated at 95℃ for 15min, centrifuged at 12 000×*g* for 20min and the supernatant was transferred to a 0.5ml micro-centrifugation tube and assayed for bioactivity.

Stimulation assay of hepatocyte proliferation. Cells were counted and adjusted to 5 × 104 cell/ml, and 100μl of cell suspensions were inoculated into the 96-well plastic plates and incubated for 6 h at 37℃ at a humidified 5% CO_2 atmosphere, the medium was then replaced by a fresh one such as human partially purified HSS, translated components, culture supernatant or cytosolic fraction of transfected cos-7 cells. After 24 h of culture, 0.5μCi of [^{3}H] thymidine per well was added and incubated for 3 h, and the cells were then collected to filters, and radioactivity was determined by a liquid scintillation counter. The activity was estimated by stimulation index (SI). SI=cpm of experimental cells/cpm of control cells.

RESULTS AND DISCUSSION

A total of 22 500 μg RNA was obtained from human fetal liver tissues and pooled. Of this RNA, 22 000 μg was processed by oligo(dT)^{a2}cellulose column chromatography and 420 μg mRNA was yielded. This mRNA was then size-fractionated by ultracentrifugation on a continuous sucrose density gradient (5%-25%), and separated into 18 fractions, and the aliquots of each fraction were translated in vitro as described in the section of methods, and its products for biological activity were tested. As shown in Figure 1, the translated products of mRNA in fraction 8 and 9 could produce a two-fold increase in the incorporation of ^{3}H-TdR into DNA of SMMC-7721 hepatoma cells.

Hepatoma cells are common targets for bioassay of hepatic stimulatory activity[9]. In the present experiment, human HSS directly prepared from fetal liver cells according to the previously described method[10] produced a two-fold increase in the incorporation of ^{3}H-TdR into DNA of SMMC-7721 hepatoma cells. Although the stimulating activity was not observed when the translated products of unfractionated mRNA was tested (Table 1), the dose-response effect of the translated products of fraction 8 and 9 mRNA (specific mRNA) on the stimulation of DNA synthesis of SMMC-7721 cell showed a positive relationship (Figure 2). The failure in detecting stimulating activity of total translated products directed by unfractionated mRNA was similar to that of our previous experiment, which might be caused by the presence of cell growth inhibitors[6].



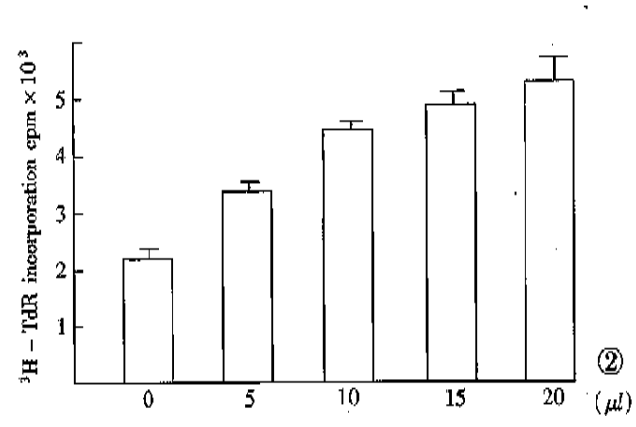


Figure 1 Sucrose density gradient centrifugation analysis of HSS mRNA.

Figure 2 Dose-response effect of SMMC-7721 cells to translated product of specific mRNA. Data are presented as $\bar{x}\pm s$ and based on the result of five experiments (four samples for each).

Table 1 Effect of translated products directed by different mRNA fractions on DNA synthesis of SMMC-7721 hepatoma cells

Fractions	cpm	SI
Control	2450±452	
Purified hHSS	5384±602	2.1[a]
Unfractionated mRNA	2430±292	1.0
Fraction 6	2202±389	0.9
Fraction 8	5013±382	2.0[a]
Fraction 9	4123±452	1.7[a]
Fraction 10	1894±172	0.7

Data are presented as x±s and based on the results of four experiments (four samples for each). [a]$P < 0.05$, showing stimulating effect.

Table 2 Organ specificity of translated products of specific mRNA ($\bar{x}$±s)

Test system	Control	Translated product of specific mRNA	Purified hHSS
SMMC-7721	1245±274	4703±236[a]	4279±193[a]
HFL	346±79	758±102[a]	693±87[a]
HFK	308±74	298±70	378±92
HFS	248±63	304±74	238±75
K562	2438±256	2058±217	3072±287
HL-60	4308±362	4703±298	3994±304

HFL, human fetal liver primary culture; HFK, human fetal kidney primary culture; HFS, human fetal spleen primary culture; hHSS, purified human hepatic stimulation substance. [a]$P < 0.05$, showing stimulating effect.

Table 3 Heat resistance of translated products of specific mRNA ($\bar{x}$±s)

Treatment	Translated product of specific mRNA (fraction 8, 9)		Purified hHSS	
	cpm	SI	cpm	SI
Control	2034±148		1876±138	
NO heating	4703±236	2.3	4279±193	2.2
65℃ for 10min	4306±287	2.1	5268±294	2.9
95℃ for 10min	4579±263	2.2	5438±306	3.0

Data are based on the results of three experiments (four samples for each). The target cell was SMMC-7721 hepatoma cell.

To rule out the possible existence of the known nonspecific stimulators, we determined the target specificity and heat-resistance of the translated product of specific mRNA. Table 2 shows that the bioactivity of translated product was liver-specific, i.e., only stimulating liver cell DNA synthesis, rather than spleen, kidney, HL-60 and so on. Table 3 indicates that the translated products were resistant to heating.

The effect of the various stimulators such as interlukin-6, fibroblast growth factor and insulin-like growth factor on the stimulation of hepatic DNA synthesis have been reported, but these stimulators could stimulate not only hepatocytes or hepatoma cells but also HL-60, K-562 cells. Unlike these sti-mulators, the translated products of fractionated mRNA were a liver-specific stimulator. The fact that translated stimulator of specific mRNA is resistant to heating and shows organ-specificity of action, strongly suggests that the partially purified human HSS mRNAs have been obtained. Because of the failure in amino acid sequence determination of HSS, the enrichment and fractionation of human HSS here should greatly facilitate the cloning of HSS cDNA using functional screening.

REFERENCES

1 LaBreque DR. Hepatic stimulator substance: discovery, characteristics and mechanism of action.Dig Dis Sci, 1991;36(5):669-673
2 Tu Q, Wu CT. Biological activities of hepatocyte growth factor of human fetal liver origin.Chin J Applied Physiol, 1990;6(3):199-203
3 Yao Z, Yang W, Zhang W, Chen Y, Yang F. Human hepatic regenerative stimulatorsubstance: partial purification and biological characterization of hepatic-stimulator substance from human fetal liver cell.Hepatology,1990;12(3): 1144-1151
4 Tu Q, Wu CT. Experimental studies on human hepatocyte growth factor of fetal liver organ in the treatment of acute hepatic failure in mice. Chin J Pathol,1991; 7(6):554-557
5 Tu Q, Wu CT. Studies on the purification and biological peculiarities of human hepatocyte growth factor of human fetal liver origin. Chin J Pathol, 1991;7(5): 537-541
6 He FC, Wu CT, Tu Q, Xing GC. Human hepatic stimulator substance: a product of gene expression of human fetal liver tissue. Hepatology, 1993;17(2): 225-229
7 Chomczynsky P, Sacehi N. Single-step method of RNA isolation by acid guanidiom thiocyanate-phenol-chloroform extraction. Anal Biochem,1987;162(1): 156-159
8 Buell GN, Wickens MP, Payvar F, Schimke RT. Synthesis of full length cDNAs from four partially purified oviduct mRNAs. J Biol Chem,1978;253(7): 2471-2482
9 LaBrecque DR, Wilson M and Fogerty S. Stimulation of HTC hepatoma cell growth in vitro by hepatic stimulator substance (HSS). Exp Cell Res,1984;150 (2):419-422
10 LaBrecque DR, Pesch LA. Preparation and partial characterization of hepatic regenerative stimulator substance (SS) from rat liver.J Physiol,1975;248(1): 273-284

Inhibitory effects of two oligosaccharides on murine melanoma experimental liver metastasis*

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Subject headings liver neoplasms, experimental; melanoma; oligosaccharides; neoplasm metastasis; disease models, animal

Abstract

AIM To observe the effects of a chemically synthesized tetrose and a natural yeast mannan on experimental liver metastasis of mouse melanoma.

METHODS After treated with 4mg tetrose (tetrose group) or 4mg mannan (mannan group) for 30 minutes at 37℃, 0.5ml 1×10^6 B16-MBK melanoma cells were injected into the spleen of mice. Fifty-five days later, melanoma metastatic nodes on the surface of the liver and in other organs as well as mouse survival time were observed.

RESULTS Of the 6 mice in control (B16 cell+PBS) group, 4 died naturally within 55 days, and 2 were killed on the 55th day. All of the 6 mice had metastases in livers, the total number of the melanoma nodes on each liver surface ranged from 2 to 30, with the largest one merging into the whole liver. One mouse had a neoplasm in the remnant site of injection, and 3 had metastases in lungs. In contrast, of the 6 mice in tetrose group, only one died on the 50th day after injection, with 3 metastases in the liver, the largest being 10 mm in diameter, the other 5 mice survived until being dissected on the 55th day after injection and had no liver metastasis, but 3 of them had neoplasms in their remnant sites of injection. In mannan group, all of the 6 mice survived and no metastasis was seen except for 2 liver nodes in one mouse with the largest diameter of 1 mm. Neither tetrose nor mannan group had metastasis out of the liver, and the weight of liver in the two groups was significantly lower than those in the control group.

CONCLUSION Both tetrose and mannan had the effects of preventing melanoma cells from experimental metastasis to and out of the liver, and prolonging the survival time of the mouse.

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INTRODUCTION

The saccharide structures on the surface of tumor cells, particularly of the highly metastatic cancer cells, have certain peculiarities, and may play vital roles in the process of metastasis. We have demonstrated that some glycopeptides can significantly inhibit experimental metastasis of mouse melanoma and Lewis lung carcinoma cell lines towards the lungs and livers[1-3], and the antimetastatic effects of the glycopeptides lie in their carbohydrate moieties. In order to explore the structural peculiarity associated with the metastasis-blocking function, and to develop new antimetastatic drugs, we designed and chemically synthesized a tetrose with a special structure, and chose the natural mannan to compare the effects of carbohydrate structures on experimental liver metastasis of mouse melanoma cells.

MATERIALS AND METHODS

Animals and reagents

Eighteen male Balb/c mice with similar body weight, obtained from the Animal Center of Beijing Medical University, were divided randomly into control group, tetrose group and mannan group, respectively, with 6 mice in each group. B16-MBK melanoma cell line (B16 cell for abbreviation) was provided by the Department of Cell Biology, Basic Medical Research Institute of Chinese Academy of Medical Sciences. Chemically synthesized tetrose was produced by the Department of Organic Chemistry in Beijing Medical University. RPMI 1640 was provided by JR Scientific Company, USA. Other reagents were A.R. or C.P. grade made domestically.

Methods

Induction of liver metastases. Lafremiere's method was adopted[4]. Briefly, with the use of sterile instruments and gloves, Balb/c mice, after anaesthetized by ether inhalation, were set in the right lateral position, and a 1-cm incision was made at the left subcostal region. The spleen was gently retracted, and the short gastric vessels and gastrosplenic ligament at the upper pole of the spleen were identified, ligated and cut, thus freeing the spleen at its pedicle. This procedure allowed the spleen to be exposed outside the abdominal cavity. B16 cell suspension 0.5 ml (2×10^6 cells/ml) in serum-free 1640 medium was injected through a 27-gauge needle positioned in the spleen through its upper pole. The

spleen pedicle was clipped with a medium hemoclip, then the spleen was removed, and the spleen pedicle repositioned intraperitoneally. The abdominal cavity was closed in one layer. The animals were fed with standard mouse chow and water ad libitum. Fifty-five days later, the mice were killed and their livers were harvested, the tumor metastasis nodes were counted on the surface of the livers.

Treatment of the B16 cells

Fourty-eight hours after passage culture, the B16 cells were detached by brief digestion with 0.125% trypsin (in PBS without Ca and Mg), the digestion was stopped by 1640 medium with 10% fetal bovine serum and then the cells were resuspended in serum-free 1640 medium, readjusted to the cell concentration at 2×10^6/ml. After incubated respectively with 4mg tetrose (tetrose group) or 4mg mannan (mannan group) for 30 minutes at 37℃ in a humidified air atmosphere of 5% CO2, the cell viability was assessed > 95% by 0.2% trypan blue exclusion test, and the cells were injected as described above in the spleen. In the control group, the cells were treated with PBS substitutionally.

Statistical analysis

The significance of differences among groups was determined by the Student's *t* test or χ^2 test. Two-tail *P* values were presented for all experiments.

RESULTS

The melanoma experimental hepatic metastasis model

With 1×10^6 cells injected intraspleen, 4 mice in the control group died naturally within 55 days after the injection of B16 cells, and 2 mice were killed on the 55th day (Figure 1). All the mice in the control group had metastases in the livers, the number of the metastastic nodes ranged from 2 to 30, with the largest one merged into the whole liver. The liver weights were 3.8 g ± 1.5 g. Of the 6 mice, 3 had lung metastases simultaneously and one mouse was found to have a neoplasm in the injected area.

The effects of oligosaccharide on melanoma liver metastasis

Tetrose group. One of the 6 mice died on the 50th day of injection with 3 melanoma nodes on its liver surfaces, the largest diameter was 10mm, the other 5 were killed on the 55th day, 3 of which had neoplasms in their remnant sites of injection, but without metastases either in or out of the livers. The weights of livers (1.4 g ± 0.1 g) were significantly lower than the control group (Table 1).

Mannan group. All of the 6 mice survived until the dissection day, and only one mouse had 2 metastases in the liver with the node diameters < 1mm, others had no metastases either in or out of the livers. The livers weighted 0.85 g ± 0.02 g, being significantly lower than the control ($P<0.05$).

DISCUSSION

In our experimental liver metastasis model, intrasplenic injection of B16 cells was used to induce the cells into portal circulation, and allow the tumor cells to form liver metastases. The fact that all mice in the control group developed metastases in the livers indicated that the model was highly reproducible, carried a high metastatic rate, and therefore was a preferable model for experimental liver metastasis. Complete ligation of the gastric and other vessels and prevention of direct spleen clamping were critical, and leakage should be avoided, if it appears, the mouse should be given up.

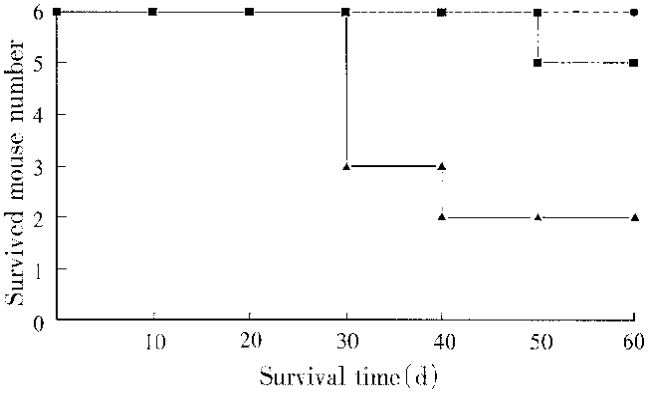

Figure 1 Mouse survival curve in each group after injection of B16 cell. -▲- Control ···●··· Mannan –■– Tetrose

Table 1 Effects of two oligosaccharides on experimental liver metastasis of B16 cells

Groups	Total No. mice	Liver metastases			Metastases out of liver (mice No.)
		Mice No.	Node No.	Node max. diameter	
Control	6	6	2-30	Fused to whole liver	3△
Tetrose	6	1[a]	0-3	10mm-8mm	0[a]
Mannan	6	1[a]	0-2	<1mm	0[a]

χ^2 test [a]$P<0.05$ *vs* control; △: metastases were found in lungs.

Recently, Dean *et al*[5] successfully inhibited experimental metastasis to lungs using synthetic multivalent lactosyl clusters. Tsukada *et al*[6] proved the antimetastatic and growth inhibitory effects of N-acetylchitohexaose in mouse Lewis lung carcinoma. The tetrose and mannan in the present study, with the structure differing from the above two carbohydrates, are strikingly effective in inhibiting experimental liver metastases. The tetrose we designed exists in the saccharide chains of tumor cell surface, its action suggested that the carbohydrate with this structure may block the metastatic process of tumor cells. The antimetastatic effect of mannan is also inspiring. Chandrasekaran *et al*[7] demonstrated *in vitro* that the spreading of B16 cells on basement membrane depends on the N-linked high mannose carbohydrate structure. The present study showed that after incubated with mannan, the B16 cells were blocked to metastasize toward the liver,probably by the blocking effect of mannan on the interaction, including spreading of B16 cells and basement membrane. As we know, spreading is a prerequisite of a series of biological processes (such as secreting proteinase, migration and proliferation) in invasion of tumor cells.

It should be noted, however, that the tetrose suppressed experimental liver metastases without inhibiting the neoplasia, since there were neoplasms in the remnant sites of injection in some mice in the tetrose group. The findings also proved that the antimetastatic effect was not due to the cytotoxic effect of the oligosaccharides, as all cells were fully viable after the treatment with tetrose or mannan. All these demonstrated that these two oligosaccharides selectively inhibited the experimental metastasis of mouse melanoma cells.

REFERENCES

1 Zhao Y, Zhou RL. The anti-metastatic effect of laminin glycopeptides in mouse B16-MBK melanoma experimental metastasis. *J Beijing Med Univ*,1992;24 (5):404-406
2 Zhang QY, Zhou RL, Zhang CY. The pathological observation of anti-metastatic effect of glycopeptides in mouse Lewis lung carcinoma experimental metastasis. *J Biochem*,1991;7(6):719-721
3 Liu YP, Zhou RL, Zhang S. Laminin glycopeptides inhibit mouse melanoma liver metastasis.*J Beijing Med Univ*, 1996;28(2):91-92
4 Lafremiere R, Rosenberg SA. A novel approach to the generation and identification of experimental hepatic metastasis in a murine model. *JNCI*,1986;76(2):309-315
5 Dean B, Oguchi H, Cai S, Otsuji E, Tashiro K, Hakomori S et al. Synthesis of multivalent β-lactosyl clusters as potential tumor metastasis inhibitors.*Carbohydrate Res*,1993;245:175-192
6 Tsukada K, Matsumoto T, Aizawa K. Antimetastatic and growth-inhibitory effects of N acetylchitohexaose in mice bearing Lewis lung carcinoma. *Jpn J Cancer Res*,1990;81(2):259-263
7 Chandrasekaran S, Tanzer ML, Giniger MS. Oligomannosides initiate cell spreading of LN-adherent murine melanoma cells. *J Biol Chem*,1994;269(5): 3356-3366

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Detection of hepatoma cells in peripheral blood of HCC patients by nested RT-PCR*

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Subject headings liver neoplasms; carcinoma, hepatocellular; RNA, messenger/blood; alpha-fetoproteins polymerase chain reaction; neoplasm metastasis

Abstract

AIM To offer a more simple method with a high sensitivity and specificity for detection of hepatoma cells in peripheral blood of the patients with HCC.

METHODS Improved nested RT-PCR method was used to detect the expression of AFP mRNA in nuclear cells separated from peripheral venous blood.

RESULTS AFP mRNA contained in ten hepatoma cells was detected from 2mL peripheral blood.

CONCLUSION The improved nested RT-PCR assay for AFP mRNA expressed in cancer cells in peripheral blood might be a valuable method for clinical diagnosis of HCC.

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INTRODUCTION

The metastatic potential of hepatocellular carcinoma (HCC) is not only directly correlated with prognosis of the patients with HCC, but also extremely valuable for selection of adequate therapies for HCC. Detection of circulating hepatoma cells in peripheral blood might be an indicator of metastatic potential of hepatocellular carcinoma. Human α-Fetoprotein (AFP) is a well-known marker for hepatoma cells. Although the serum AFP level could be one of the useful biochemical indicators for diagnosis of HCC, the conventional measurements for serum AFP concentration is not so sensitive to identify an extremely small amount of cancer cells in peripheral blood. Based on the nested RT-PCR method for detecting AFP mRNA in the circulation reported recently[1,2], we merged the reverse transcription reaction and the first PCR into a single step, and then combined it with nested PCR. By this way, AFP mRNA contained in 10 human hepatoblastoma cells (HepG-2) could be detected in 2mL peripheral venous blood. Being highly sensitive and specific, the improved method requires relatively a small amount of blood sample, and less time to obtain similar satisfactory results.

MATERIALS AND METHODS

Materials

HepG-2 was derived from the Institute of Virology, Chinese Academy of Preventive Medical Sciences. The AFP primers[3] purified by HPLC were obtained from Cybersyn, BJ. The external primers were: 5' ACT GAA TCC AGA ACA CTG CAT AG3' and 5' TGC AGT CAA TGC ATC TTT CAC CA3'. The internal primers were: 5'TGG AAT AGC TTC CAT ATT GGA TTC3' and 5'AAG TGG CTT CTT GAA CAA ACT GG3'. Taq DNA polymerase and AMV reverse transcriptase were purchased from Sino-American Corp. and Promega Inc., respectively.

Methods

Separation of nuclear cells from peripheral blood. Fresh healthy peripheral venous blood was collected into a sterilized tube containing 0.5% EDTA. One thousand HepG-2 cells were mixed with 2mL healthy blood and serial dilution of cancer cells was then prepared. Each dilution sample contained 1000, 100, 10 and 1 HepG-2 cells per 2mL of blood, respectively. Nuclear cells were separated from peripheral venous blood using routine method by Ficoll

isolation.

Extraction of total RNA from nuclear cells. Total RNA of nuclear cells separated from whole blood was extracted using single-step method by acid guanidinium thiocyanate extraction[4].

Reverse transcription and the first PCR. A total amount of 50μL reaction solution contained 5μL 10 ×PCR buffer, 0.2mmol/L of each dNTPs, 20 pmol-50pmol of each external primers, 50 U RNAase inhibitor, 30 U AMV reverse transcriptase, 3U Taq DNA polymerase and RNA template. The mixture solution was incubated at 42℃ for 30min, and followed by initial denaturation at 94℃ for 2min. The PCR procedure was carried out as follows: denaturation at 94℃ for 30s, annealing at 52℃ for 30s and extension at 72℃ for 40s, and the thermal cycles were repeated 32 times.

Nested PCR. Reaction mixture of 50μl contained 5μL of 10×PCR buffer, 0.2mmol/L of each dNTPs, 20pmol-50pmol of each internal primers, 3U Taq DNA polymerase and 10μL of the first PCR product. The PCR procedure was: denaturation at 94℃ for 30s, annealing at 52℃ for 30s and extension at 72℃ for 40s; and the cycles were repeated 32 times as well.

Gel electrophoresis. The final amplification product was electrophoresed on 2% agrose gel and stained with ethidium bromide for the specific band of 101bp.

Internal standard control assay. Total RNA extracted from HepG-2 cells was quantified and diluted serially with DEPC-treated water, which was used as the internal standard control of this assay. Each of internal standard control samples contained 5, 1, 0.5, 0.05, 0.005, 0.0005 and 0.00005 μg of RNA template, respectively. The diluted samples were applied to nested RT-PCR amplification following the same procedure described above.

RESULTS

The amplification template used in this method was total RNA derived from HepG-2 cells. To seek a rapid and simple detecting method for AFP mRNA expression in hepatoma cells, we merged the reverse transcription reaction and the first PCR amplification into a single step and then combined with nested PCR. Gel electrophoresis analysis showed that specific bands (101bp) of AFP mRNA were not observed in nuclear cells from healthy peripheral venous blood. When 1000, 100, 10 HepG-2 cells were added into 2mL blood, specific bands of 101bp were demonstrated in all RNA samples from nuclear cells in each of diluted samples. However, RNA from the diluted blood containing 1 HepG-2 cells was insufficient for detection (Figure 1).

As indicated in Figure 2, internal standard control assay demonstrated that the intensity of the specific bands for AFP mRNA was significantly correlated with the amount of total RNA templates. No specific bands for AFP were detected in the sample of 5×10^{-6}μg RNA from HepG-2 cells by nested RT-PCR. With regard to negative control with absence of RNA from HepG-2 cells, it was also impossible to detect such specific bands.

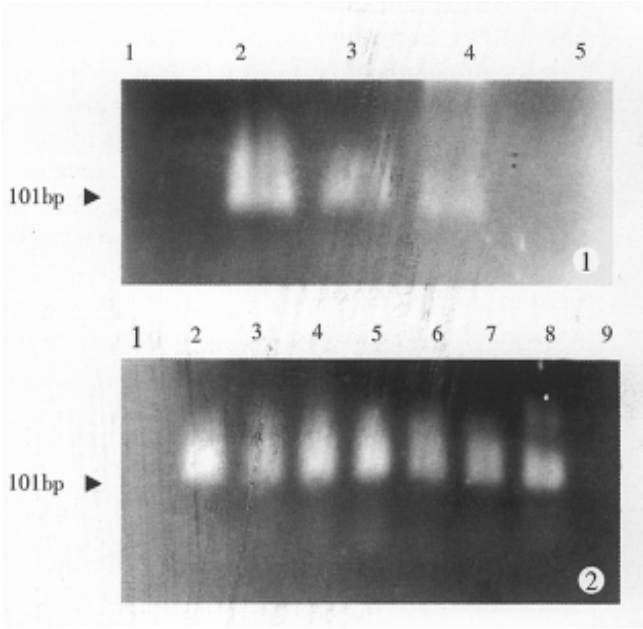

Figure 1 Amplification of AFP mRNA expressed in nuclear cells in peripheral blood by nested RT-PCR.
Lane 1: Blood from healthy volunteers.
Lanes 2-5: Titration of HepG-2 cells. Each lane includes (from left to right) 1000, 100, 10, 1 HepG-2 cells in 2 mL normal blood, respectively.
Figure 2 Amplification of AFP mRNA in HepG-2 cells by nested RT-PCR.
Lane 1: Negative control.
Lanes 2-9: Titration of total RNA extracted from HepG-2 cells. Each lane includes (from left to right) 5, 1, 0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000005μg total RNA, respectively.

DISCUSSION

Primary hepatocellular carcinoma is one of the most common malignant tumors in China, with the death rate ranking the first in the urban population and the second in rural areas of China. Prognosis of HCC is not only closely associated with early diagnosis and optimal therapy, but also with some important characteristics of hepatoma cells, such as the potential of intrahepatic metastasis, extrahepatic metastasis and recurrence. How to estimate the metastatic potential of hepatoma cells has remained a major puzzle for many years. AFP is expressed by

hepatoma cells and then secreted into circulating blood, thus being regarded as a useful marker for hepatocellular carcinoma. Hepatoma with the potential of intrahepatic or extrahepatic metastasis often shed hepatoma cells into portal venous blood and peripheral blood.

It was reported that the human albumin mRNA could be used as a marker of circulating hepatocytes in HCC[5], and that AFP mRNA expression could be detected in a tiny amount of hepatoma cells in circulating blood of patients with HCC[1,2] by nested RT-PCR. Furthermore, they found that the level of AFP mRNA expression was significantly correlated with the prognosis of HCC. RT-PCR is a very sensitive technique for amplifying nucleic acids, which could be often used to detect tiny amounts of mRNA copies. Since nested RT-PCR utilizes a couple of internal primers to reamplify the specific PCR product, it exhibits higher sensitivity, stronger specificity and lower false positive occurrence as compared to single RT-PCR.

In this study, we attempted to provide a more simple nested RT-PCR method with high sensitivity and specificity for detecting a tiny amount of hepatoma cells in circulating blood of patients with HCC. By this method, AFP mRNA could be detected when 10 HepG-2 cells were present in 2mL peripheral blood or portal venous blood, suggesting that the sensitivity of this method is similar to that reported in literature. Thus, it might be a desirable assay for clinical evaluation of metastatic potential of hepatocellular carcinoma.

REFERENCES

1 Komeda T, Fukuda Y, Sando T, Kita R, Furukawa M, Nishida N *et al.* Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. *Cancer*, 1995;75(9):2214-2219
2 Matsumura M, Niwa Y, Hikiba Y, Okano K, Kato N, Shiina S *et al.* Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. *Biochem Biophy Res*, 1995;207(2):813-818
3 Morinaga T, Sakai M, Wegmann TG, Tamaoki T. Primary structures of human a-fetoprotein and its mRNA. *Proc Natl Acad Sci USA*, 1983;80(15): 4604-4608
4 Chromezynski P, Acchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt Biochem*, 1987;162(1): 156-159
5 Hillaire S, Barbu V, Boucher E, Moukhtar M, Poupon R. Albumin messenger RNA as a marker of circulating hepatocytes in hepatocellular carcinoma. *Gastroenterology*, 1994;106(1):239-242

Hepatic adenylate energy charge levels in patients with hepatoma after hepatic artery embolization*

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Subject headings ketone bodies; energy metabolism; liver/metabolism; liver neoplasms/therapy; carcinoma, hepatocellular/therapy; embolization, therapeutic; postoperative complication; liver function test

Abstract

AIM To evaluate hepatic energy charge levels of the patients with hepatoma after hepatic artery embolization and its relation to postoperative complications.

METHODS Sixty-nine patients with hepatoma were continuously measured for their arterial blood ketone body ratio (AKBR) and compared with their postoperative clinical course or conventional liver function test after various hepatic artery embolization.

RESULTS AKBR in high radiation dose or jaundice group drastically decreased at 1-3 days and recovered slowly. Patients were classified into three groups according to the value of AKBR: group A (35 cases), AKBR remained higher than 0.7; group B (31 cases), AKBR had transiently dropped to 0.4-0.7 and then increased to preoperative value; and group C (3 cases), AKBR decreased steadily to below 0.4. The occurrence rate of various complications were 5.7%, 32.3% and 100% in the three groups, respectively ($P < 0.005$).

CONCLUSION The AKBR which reflects hepatic mitochondria redox state is morereliable as a direct indicator to assess hepatic tolerance for embolization than routine liver function test.

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INTRODUCTION

Hepatic artery embolization has been the first choice for advanced hepatomas in recent years, the therapeutic effect depends upon not only thoroughness of chemoembolization or radioembolization, but also closely correlated with liver function reserve[1]. It has been shown that conventional liver function tests could not give a direct and accurate evaluation of postoperative metabolic abnormality, while the arterial blood ketone body ratio (AKBR) which reflects hepatic mitochondria redox state could serve as a relatively more reliable parameter to assess the extent of hepatic injury and vitality[2]. This study investigates the effects of various embolization methods on hepatic energy charge level and its relation to postoperative complication.

MATERIALS AND METHODS

Subjects

Sixty-three patients with unresectable primary hepatocellular carcinoma, 3 metastatic liver carcinoma and 3 haemangioma confirmed by B-type ultrasonography, hepatic angiography, computed tomography and operation were included in this study. There were 66 males and 3 females, aged from 21 to 64 years with a mean age of 44.3 years. Liver cirrhosis was found in 62 patients (89%). Preoperative child classification of liver function was A stage, 53 cases; B stage, 15 cases; and C stage, 1 case and increased total bilirubin was detected in 20 patients.

Hepatic artery embolization

Intraoperative transcatheter arterial embolization was accomplished via selective right hepatic artery (39 cases) or nonselective common hepatic artery (30 cases). Eight patients received simple embolization with Gelfoam stripes. Chemoembolization with emulsion of lipiodol 10mL-15mL containing adriamycin 20mg and mitomycin C 10mg was applied to 30 patients. Radioembolization was performed on 31 patients using phosphorus 32 glass radiomicrospheres provided by the Chinese Academy of Atomatic Energy. The microspheres of 0.75mg-5.6mg (radioactivity, 2.16mCi/g-11.78mCi/g, mean 61 μm) was equal to a mean 33Gy of hepatic absorbed radiation doses used in this study.

AKBR assay

For the assay of ketone bodies, 4?ml arterial blood

samples were obtained with a heparinised syringe via the femoral artery 1 day preopratively, and 1, 3, 5 and 7 days postoperatively. When blood samples were taken, all patients were receiving intravenous infusion with 10% glucoses to maintain their blood glucose levels at 6.7mmol/L - 11.2mmol/L. The blood samples were immediately mixed with the same volume of ice-cold 10%w/v perchloric acid. The suspension was centrifuged at 10 000× *g* for 15min at 0℃. The supernatant was adjusted to a pH of 7.0 with cold 65%w/v potassium carbonate and recentrifuged at 10 000× *g* for 5min at 0℃. The final supernatant was used to measure the AKBR (AKBR = acetoacetate/ β-hydroxybutyrate), spectrophotometrically by the standard methods of Mellanby and Williamson[3].

Statistical analysis

The AKBR of intergroups was compared by analysis of variance and the positive rates by Chi-square test. *P* value < 0.05 was considered to be statistically significant.

RESULTS

As shown in Figure 1, the patients were classified into three groups according to the postoperative changes in their AKBR. In group A (35 patients), the AKBR was above 0.7 after operation and returned to near the preoperative level in 3-7 days. In group B (31 patients), the AKBR remained between 0.4 and 0.7 after operation and thereafter gradually rose to above 0.7. In group C (3 patients), the AKBR decreased to below 0.4 and even dropped to below 0.25 without restoration.

The major postoperative complications in each group are summarized in Table 1, the occurrence rate was 5.7% (2/35), 32.3% (10/31) and 100% (3/3) respectively (P£¼0.005), with no significant statistical difference among the groups (*P*<0.005). However, no difference was found in postoperative changes in serum enzyme activities, albumin and total bilirubin concentrations among the three groups.

As shown in Table 2, the radioembolization at large doses had marked effect on the AKBR levels, which declined rapidly after operation and recovered slowly along with repeated occurrence of complications. The preoperative AKBR in the group with elevated bilirubin was lower than that of normal bilirubin ($P < 0.02$), and most of the cases fell to below 0.7 within 1-3 postoperative days and were accompanied by a higher morbidity of complications. Liver dysfunction and hemorrhage occurred in six patients with low preoperative AKBR level (0.69-0.85), and two of them eventually died of multiple organ failure, whereas only two of other fourteen patients with AKBR of 0.97-1.43 preo- peratively suffered from cholecystitis and wound dehiscence.

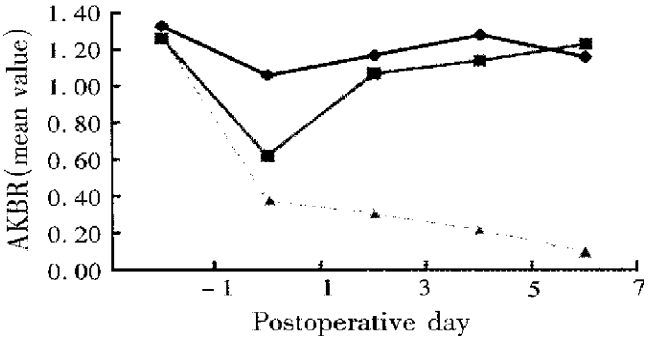

Figure 1 Postoperative changes of AKBR in three groups.

Table 1 The relationship between changes in their AKBR and postoperative complications

Groups	Simple embolization			Chemoembolization			Radioembolization		
	Cases	Complications	Class	Cases	Complications	Class	Cases	Complications	Class
A	7	0	0	15	1	2	13	1	2
B	1	0	0	14	3	4	16	7	6
C	0	0	0	1	1	5	2	2	7

The class of complication includes: liver dysfunction, hemorrhage, multiple organ failure, cholecystitis, wound dehiscence, coagulation defects, pulmonary infection, renal insufficiency, pancreatitis, enteritis and sepsis.

Table 2 Effects of radiation doses, embolic area and with or without jaundice on the AKBR

Groups	Cases	AKBR value					Complications
		Preoperation	First day	Third day	Fifth day	Seventh day	
High doses	8	1.26±0.41	0.66±0.15[a]	0.76±0.24[a]	0.81±0.18[a]	0.71±0.29[a]	63[b]
Moderate doses	12	1.21±0.39	0.97±0.26[a]	1.09±0.15[a]	1.17±0.12[a]	1.11±0.23[a]	25
Low doses	11	1.23±0.25	0.89±0.12	0.98±0.19	1.13±0.20	1.08±0.11	18[b]
With jaundice	20	0.99±0.26[a]	0.72±0.19[a]	0.84± 0.20[a]	0.91±0.32[a]	0.89±0.21[a]	40[b]
Without jaundice	49	1.36±0.31[a]	0.92±0.18[a]	1.07±0.13[a]	1.08±0.25[a]	1.18±0.23[a]	15[b]
Inselective	30	1.26±0.24	1.01±0.32	1.03±0.25	1.18±0.11	1.03±0.29	27
Selective	39	1.33±0.20	0.98±0.41	1.12±0.10	1.04±0.27	1.16±0.18	18

Intergroup comparison: [a]*P*<0.05, [b]*P*<0.05. High doses: mean 55Gy, Moderate doses: 31Gy, Low doses: 18.5Gy.

DISCUSSION

AKBR can reflect the oxidoreduction state of free nicotinamide adenine dinucleo tides (NADH) in liver mitochondria, i.e., AKBR=K × free NAD^+/ free NADH, which is closely linked to energy charge level (ATP + 1/2ADP/ATP + ADP + AMP)[4]. The AKBR, when it is greater than 1, 1 -0.7, 0.7-0.4, 0.4-0.25 and less than 0.25, may indicate respectively normal mitochondria function (takes mainly sufficient glucose oxidation with an increased NAD^+), slight mitochondria dysfunction (takes preferentially oxidized fatty acids as an efficient energy source rather than glucose such as group A), mitochondria membrane impairment (leads to inhibited pyruvate oxidation and enhanced β-oxidation of long-chain fatty acid, various complications may occur under the condition of constant ketogenesis, such as group B), serious mitochondria injury and death (results in an irreversible damage of the energy-generating system such as group C).

It has been found that acute ischemia, chemical toxic agent and nuclide internal radiation precipitate direct injury to mitochondria. Our results indicate that the reduced AKBR was related to radiation doses, and the radiomicrosphere affected obviously the hepatic energy charge level among the three therapeutic patterns (embolization, chemoembolization, radioembolization) and the two targets (selective or inselective). Hepatocellular hypoxia could inhibit the electron transport system in mitochondria chain and produced excessive accumulation of NADH. The range of AKBR decline has proved to be paralleled to the blocked extent and time of hepatic blood flow[5], and the hepatic cell can be compromised by β-ray radiation and terminal embolic material. Therefore, internal radiation therapy at large doses might not be an advisable procedure for patients with liver carcinomas.

Preoperative AKBR might serve as an approximate index for evaluation of energy reserve status. When patients were associated with severe cirrhosis and obstructive jaundice, chronic mitochondria dysfunction potentially preexisted because of their deprived portal flow, reduced endogenous insulin level acting on oxidative phosphorylation process, altered ATP transport rate and prohibited directly by bilirubin[2]. Therefore, the higher AKBR may indicate some acute compensatory capacity for injury, and their tolerance to embolization can be quantitatively assessed by combination of redox tolerance test (RTT)[6], while the lower AKBR demonstrates that preoperative compensatory activity for synthesizing liver ATP is up to an extreme limit, e.g. various complications were observed in six patients with hyperbilirubinemia and preoperative AKBR of 0.69 -0.85. Hence, the indication for TAE must be carefully considered in patients with those risk factors.

REFERENCES

1 Nakamura H, Mitani T, Murakami T, Hashimoto T, Tsuda K, Nakanishi K *et al.* Five year survival after transcatheter embolization for HCC. *Cancer Chemother Pharmacol*,1994;33(Suppl):89-92
2 Iwata S, Ozawa K, Shimahara Y, Mori K, Kobayashi N, Kumada K *et al.* Diurnal fluctuation of arterial ketone body ratio in normal subjects and patients with liver dysfunction.*Gastroenterology*, 1991;100(9):1371-1378
3 Williamson DH, Mellanby T. Methods of enzymatic analysis. New York: *Academic Press*, 1974:1836-1843
4 Ozawa K, Chance B, Tanaka S, Iwata S, Kitai T, Ikai I. Linear correlation between acetoace-tate / β-hydroxybutyrate in arterial blood and oxidized flavoprotein / reduced pyridinenucleotide in freeze trapped human liver tissue. *Biochim Biophys Acta*, 1992;1138(6):350-352
5 Kajimura K, Moriyasu F, Someda H, Okuma M, Mori K, Ozawa K. Transcatheter hepatic arterial drug infusion therapy for hepato-cellular carcinoma: effect on the arterial ketone body ratio. *Scand J Gastroenterol*, 1993;28(6):522-526
6 Yan LN, Wu YT, Meng XQ, Cheng LL, Li XW, Zhou Y *et al*. Redox tole-rance test: an indicator of preoperative hepatic functional reserve in hepatoma. *Asian J Surg*,1994;17(4):365-370

 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(2):153-157

Effects of combined use of diallyl disulfide and *N*-acetyl-cysteine on acetaminophen hepatotoxicity in β-naphthoflavone pretreated mice

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Subject headings diallyl disulfide; *N*-acetyl-cysteine; acetaminophen; beta-naphthoflavone; liver/drug effects; glutathione; 7-alkoxycoumarin O-dealkylase

Abstract

AIMS To assess the protective effect of diallyl disulfide (DADS) and its combined use with *N*-acetyl-cysteine (NAC) on acetaminophen (APAP) hepatotoxicity in C57BL/6N (B6) mice pretreated with β-naphthoflavone (BNF).

METHODS B6 mice were divided into six groups and all compounds used were injected intraperitoneally. Except for control and APAP group (receiving APAP only), the other groups received an injection of APAP (350mg/kg) 48 hours after BNF (200mg/kg) and either of DADS (200mg/kg), or NAC (500mg/kg) or both DADS and NAC. DADS was given 2 hours before APAP and NAC was injected with APAP. The mean survival time was recorded and livers were examined histologically. Hepatic glutathione (GSH) levels and plasma ALT were also determined at different time points. To evaluate the effect of DADS or NAC on hepatic P450 induction by BNF, liver microsomes were prepared and 7-ethoxyresorufin O-dealkylase (ERD) activity was determined using spectrofluorometrical methods. In vitro effect of DADS or NAC on ERD activity was assayed by directly incubating microsomal suspension with DADS or NAC of different concentrations.

RESULTS APAP was not toxic to mice without BNF pretreatment, but caused severe liver necrosis and death of all BNF-treated mice in 4 hours. A sharp depletion of GSH (approximately 62% of its initial content at 2 hours and 67% at 4 hours) and a linear elevation of ALT levels (536.8±29.5 Sigma units at 2 hours and 1302.5±74.9 at 4 hours) were observed. DADS and NAC given individually produced mild protection, resulting in prolonged survival, a slower decline of GSH level and a less steeper elevation of ALT level. All mice died eventually. Co-administration of DADS and NAC completely protected mice. GSH level in this group lowered by about 35% and 30% at 2 and 4 hours, and ALT was 126±18 and 157.5±36.6 Sigma units at 2 and 4 hours. ERD activity in BNF-treated mice was about 5 times that of the constitutive level determined in normal mice. Neither DADS nor NAC inhibited P450 1A1/1A2 induction as determined by their effect on the induction of ERD activity. In vitro assay indicates that DADS, but not NAC, was a potent inhibitor of ERD activity (IC_{50}=4.6μM).

CONCLUSIONS A combined use of both DADS and NAC produced full protection in BNF treated mice against APAP hepatotoxicity. The mechanism is that DADS inhibits P450 1A1/1A2 activity, but not induction, which substantially reduces production of NAPQI, while NAC enhances liver detoxifying capability via serving as a precursor of GSH and stimulating GSH synthesis.

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INTRODUCTION

Acetaminophen (APAP) has been widely used for decades, especially as an over the counter analgesic in North America. It has been long known that an overdosed APAP can cause severe damages to the liver or even death of the experimental animals and individuals who have ingested large quantities of APAP accidentally or in an attempt to commit suicide. In the liver, APAP is bioactivated and converted by cytochrome P450 to its metabolite, *N*-acetyl-p-benzoquinoneimine (NAPQI), which has been shown to be toxic to animals in vivo[1] and to cultured hepatocytes[2,3]. Although the precise mechanism of APAP hepatotoxicity is not well understood, a number of studies have suggested that NAPQI exerts a cytotoxic effect through its covalent binding to cytosolic or microsomal proteins and membrane components[4-6], inhibition of mitochondrial respiration, depletion of ATP[7,8], etc.

For bioactivation of APAP, several P450 isoforms are implicated, including P450 1A1, 2A1, 2B1, 2C11, 2E1, 3A1 and 3A2[9,10]. These enzymes are inducible by cigarette smoking, alcohol consumption, drugs, and some chemical compounds

used daily at home for cleaning or as a pesticide. Elevation of P450 activity by these inducers can markedly enhance APAP hepatotoxicity in humans[11] and experimental animals[12]. For instance, alcohol is an inducer of P450 2E1 and therefore alcoholics are more susceptible to and at higher risk for development of APAP hepatotoxicity[11]. More attention should be paid to these risk factors because these individuals may develop hepatotoxicity at the rapeutic doses. In recent years, several compounds have been tested for their protective effects on overdose APAP. However, information about the possible treatment for APAP toxicity in P450-elevated animal models or human subjects is very limited. Diallyl sulfide (DAS) is a P450 inhibitor specifically for 2E1. Recently, a study showed that DAS prevented APAP hepatotoxicity in normal rats[13]. The current work investigated the role of diallyl disulfide (DADS) and its combined use with *N*-acetyl- cysteine (NAC) in protecting APAP-caused liver damage in mice in which P450 levels were induced by β-naphthoflavone (BNF) pretreatment. To our knowledge, this is the first investigation on the inhibitory effect of DADS on hepatic P450 1A1/1A2 induced by BNF in mice and the protective effect of the use of DADS and NAC in combination on APAP caused-hepatotoxicity.

MATERIALS AND METHODS

Effect of DADS and NAC on APAP hepatotoxicity

Animals and animal treatment. C57B/6N (B6) mice (4 - 6 weeks of age, 18 g - 20 g in body weight) were purchased from Harlan Sprague Dawley (Indianapolis, USA). The mice were divided into six groups (10 mice each) and treatment for each group is shown in Table 1. All compounds used were purchased form Sigma, USA (except indicated otherwise) and injected intraperitoneally. BNF was injected at dose of 200mg/kg body weight (in corn oil) 48 hours prior to administration of APAP (350 mg/kg body weight in saline) as described previously[14]. DADS (200mg/kg body weight, purchased from Aldrich, USA) was diluted in 0.2ml corn oil and given 2 hours before APAP challenge. NAC of 500mg/kg body weight was injected at the same time when APAP was given. For control, corn oil and saline were used in replace of BNF and APAP, respectively. The animals were then kept in warm environment under close observation.

Table 1 Treatment of animals in different groups

	APAP	BNF+APAP	DADS	NAC	DADS+NAC
BNF		200	200	200	200
DADS			200		200
APAP	350	350	350	350	350
NAC				500	500

Values were doses used (mg/kg body weight)

Evaluation of APAP hepatotoxicity. After APAP challenge, the survival time for each group was recorded, calculated and expressed as mean±SD. APAP-induced hepatotoxicity in each group was also evaluated histologically and enzymatically. For histology, immediately after death of the animal or sacrifice of the mice surviving longer than 48 hours, the liver was removed, fixed in 10% neutral buffered formalin, washed with phosphate buffer saline (PBS), dehydrated in an increasing concentration of ethanol, and finally embedded in paraffin. Sections of 6 μm in thickness were stained with hematoxylin and eosin and examined under light microscopy. The extent of liver necrosis was evaluated semi-quantitatively using a scale of 0-4 according to the scoring system introduced by Mitchell *et al*[15]. Score 0, indicates no evidence of necrosis; score 1, less than 6%; score 2, 6%-25%; score 3, 26%-50%; and score 4, more than 50% of the liver cells are necrotic.

Blood samples were collected at 2, 4 and 6 hours after APAP administration and plasma ALT levels were determined using Sigma GPT Kit (following the manufacturer's instruction). The values for each group were expressed as mean ±SD. ALT less than 20 Sigma units is considered normal and a range of 20-35 units is a border line according to the manufacturer's instruction.

Effects of DADS and NAC on hepatic GSH levels

Another set of groups of B6 mice was used in order to determine the effects of DADS and NAC on hepatic GSH levels. After the same treatment as shown in Table 1, the mice were sacrificed at 2, 4, 6, 8 and 24 hours after APAP administration. The liver was homogenized in 4 volumes of 0.1M PBS and the homogenate was mixed with an equal volume of 4% sulfosalicylic acid. After centrifugation, 0.5ml supernatant was added to 4.5ml 0.1mM bis-(3-carboxy-4-nitrophenyl) disulfide in 0.1M PBS (pH 8.0). The mixture was incubated at 25℃ for 60 minutes in the dark. GSH concentration was determined at absorbance 412nm using a Shimadzu UV-Visible Recording Spectrophotometer UV-160.

Effect of DADS and NAC on elevated P450 1A1/1A2 activity induced by BNF

To explore the mechanism of DADS protection against APAP hepatotoxicity, we further investigated the effect of this compound on hepatic P450 1A1/1A2 activity induced by BNF. Five groups of B6 mice were used and treated as control, BNF-APAP, DADS, NAC, and DADS+NAC groups as summarized in Table 1, except that APAP was omitted in all of these groups and NAC was given 47 hours after BNF. Forty-eight hours after BNF, the mice were killed and livers were immediately removed for determination of activity of hepatic microsomal 7-ethoxyresorufin (ER) O-dealkylase by the method

introduced by Burke *et al*[16]. Briefly, livers were homogenized in 0.1M Tris buffer containing 0.25M sucrose (pH 7.5). The supernatant fractions from the centrifuged liver homogenate (13 500 × *g* for 20min) were recentrifuged at 105 000 × *g* for 60min. The pellets were washed and resuspended in 0.1M PBS and used for assay immediately. The reaction mixture contained 2ml PBS, 20μl microsomal suspension, and 10μl ethoxyresorufin (50μM). A baseline of fluorescence was recorded at an excitation wavelength of 510nm and an emission wavelength of 586nm using a Shimadzu fluorospectrometer RF-540. After addition of 200μl NADPH to the reaction mixture, the increase in fluorescence was recorded in air at room temperature. The specific activity of the enzyme was calculated by comparing with a standard curve.

Assessment of direct effects of DADS or NAC on induced P450 1A1/1A2 activity was conducted by incubating microsomal suspension prepared from BNF-treated mouse liver with different concentrations of DADS or NAC, respectively. Incubation was carried out at room temperature for 30min and followed by the measurement of 7-ethoxyresorufin dealkylase activity as described above.

Statistical analysis

Statistical significance was analyzed using statistical software StatView 4.1 (Jandel Scientific Inc., USA). One-way ANOVA and Student *t* test were used for difference among multiple values or between two values, respectively.

RESULTS

Effects DADS and NAC on APAP hepatotoxicity in BNF-pretreated B6 mice

APAP group showed no sign of APAP toxicity. However, BNF+APAP mice suffered from severe toxicity and all died eventually. The difference was statistically significant between the APAP group and BNF+APAP group in terms of mortality and mean survival time ($P<0.001$). The use of either DADS or NAC alone prolonged the mean survival time ($P<0.01$) but did not affect the mortality rate when comparing DADS group and NAC group with BNF +APAP group. The use of DADS and NAC in combination completely protected the animals, and the mortality rate was reduced to 0 ($P<0.001$), (Table 2).

Histological evaluation of the liver necrosis revealed that mild damage (Score 1) was found in 10% of mice receiving APAP only, and 90% of this group appeared normal morphologically. However, extremely severe hepatic necrosis was observed in BNF+APAP group, with Mitchell's score 4 for 9 mice and 3 for 1. The necrosis mainly occurred in the lobular center and in some cases, the normal lobular structures were destroyed. Although the use of either DADS or NAC (Group 4 and 5) could significantly prolong the mean survival time, Mitchell's scores of these mice were still recorded as high as 3 for 9 mice of DADS group and 8 mice of NAC group, respectively. Seven animals of DADS +NAC group were recorded histologically as score 1 (mild damage), and three as score 2. There was a statistical significance between DADS+NAC group and BNF+APAP group ($P<0.01$).

As shown in Figure 1, hepatic GSH in BNF+ APAP group sharply declined by 60% of its initial concentration in first two hours after APAP and continued to drop until all mice died at 4 hours. In DADS, NAC and DADS+NAC groups, GSH levels also dropped by about 35% in first two hours, though significantly higher than that of BNF +APAP group ($P<0.05$). At 4 and 6 hours, GSH in DADS+NAC group maintained at a significantly higher level ($P<0.01$, as compared with DADS or NAC group) and all mice survived whereas liver GSH in either DADS or NAC group continued to decline and all died about 7 hours after APAP. Plasma ALT levels of these groups are illustrated in Figure 2. ALT in BNF + APAP mice increased almost linearly, and DADS or NAC group showed moderate elevation while ALT in DADS+NAC group went up only mildly. Statistical significance ($P < 0.05$) was found between BNF+APAP group and either DADS or NAC group and DADS + NAC group, but not between DADS and NAC groups ($P>0.05$).

Effects of DADS on hepatic P450 1A1/1A2 activity induced by BNF

In vivo study on effects of DADS, NAC or DADS and NAC used in combination on hepatic P450 1A1/ 1A2 are shown in Figure 3. BNF can significantly induce P450 1A1/1A2, as determined by ERD activity, and compared with the constitutive activity in the control mice ($P<0.01$). There was no significant difference in ERD activity between BNF, DADS, NAC, and DADS + NAC groups ($P > 0.05$). However, in vitro study by direct incubation of microsomal suspension prepared from BNF-treated mouse liver demonstrated that DADS can significantly inhibit P450 1A1/1A2 activities induced by BNF, IC_{50} being approximately 4.6μM of DADS, whereas NAC showed no direct inhibitory effect on the enzyme activity (Figure 4 a and b).

Table 2 Effects of DADS and NAC on mortality and mean survival of mice treated with APAP

	Control	APAP	BNF+APAP	DADS	NAC	DADS+NAC
Mean survival time(h)		>48	3.17±0.18	6.29±0.49	7.0±0.40	>48
Mortality (%)	0	0	100	100	100	0

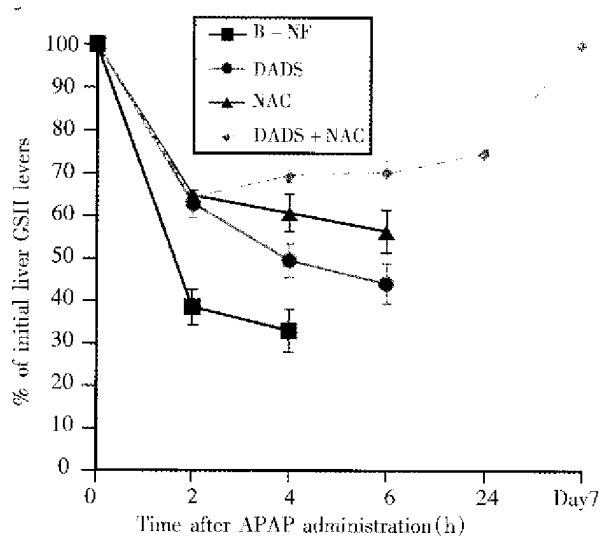


Figure 1 Effect of DADS and NAC on liver GSH level.

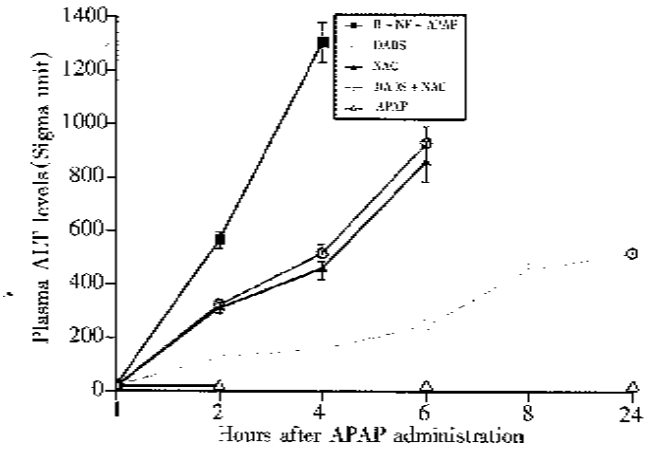


Figure 2 DADS and NAC on plasma ALT levels.

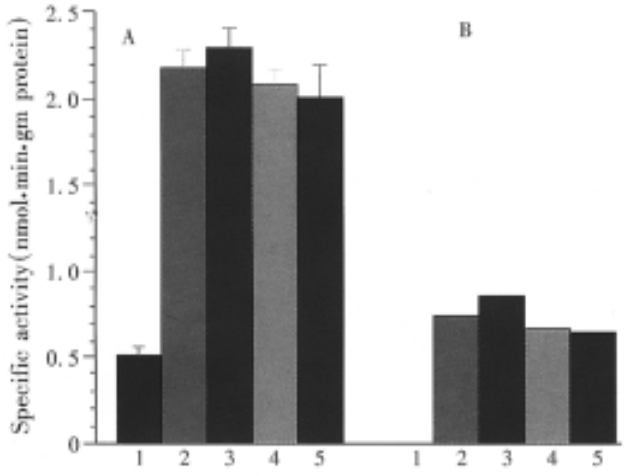
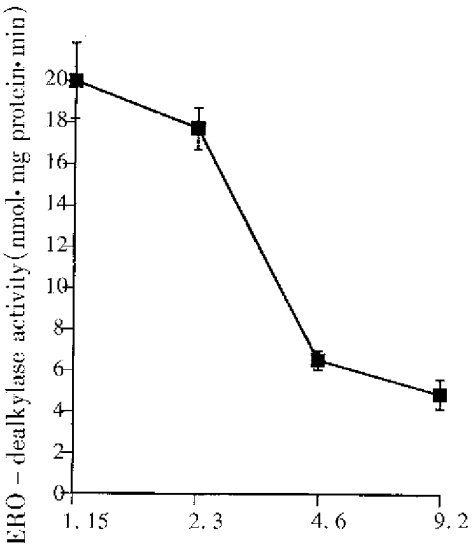


Figure 3 Effects of DADS and NAC on BNF-induced CYP-1A1/1A2 activity (O-ER activity).
1. No BNF (constitutive activity) 2. BNF (induced activity)
3. BNF+DADS 4. BNF+NAC
5. BNF+DADS+NAC
Enzyme activity assayed in absence (A) and presence (B) of anti-CYP 1A1/1A2 antibodies.



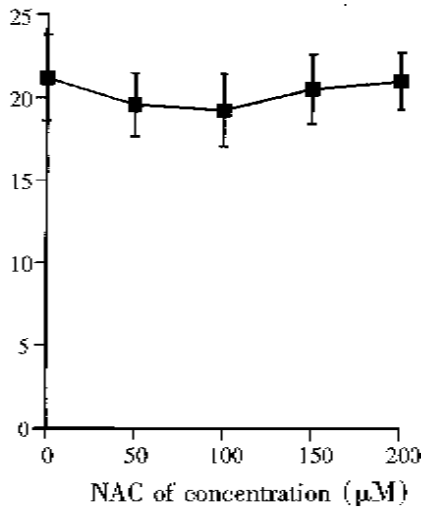


Figure 4 Direct effect of DADS and NAC on P450 1A1/1A2 activity.

DISCUSSION

Studies on APAP hepatotoxicity remain a very active area since much is still uncertain despite continuous efforts worldwide. We have previously demonstrated the inductibility of P450 1A1/1A2 by BNF in B6 mouse hepatocytes in vivo[14]. The current study showed that BNF-pretreated mice developed severe liver necrosis and all mice died in a few hours after challenged with APAP. However, when APAP of the same dosage was applied to mice without BNF pretreatment, the animals tolerated it well. These findings indicate that P450 induction by BNF in hepatocytes markedly enhanced APAP hepatotoxicity and, therefore, is a critical step in the development of liver necrosis. P450 1A1/1A2 is probably the major isoform involved in this case, which is in agreement with the study by Snawder, *et al*[12].

In treatment of APAP poisoning, compounds which inhibit P450 activity and drugs which increase

hepatic GSH pooling (i.e. enhancing liver detoxifying capability) are expected to protect animals against APAP cytotoxicity since NAPQI is converted from APAP by P450 enzymes and normally and mainly detoxified by conjugation with GSH. The reaction increases the solubility of NAPQI and facilitates its elimination through the kidneys. DADS is attractive because of its nature as an extract derived from garlic[13]. Recently, Hu[13] reported that DAS (another compound derived from garlic) given intra-gastrically at a dose of 200mg/kg body weight reduced the mortality from 40% to 0% in rats challenged with APAP at dose of 750mg/kg body weight. The protective action of DAS was thought to be related to its inhibitory effects on the hepatic P450 activity, especially for P450 2E1[16,17]. In the current study, we observed that DADS given intraperitoneally prolonged the survival time of B6 mice in which P450 activity had been induced by BNF. However, this regimen did not improve the histological evaluation and the total mortality rate, although hepatic GSH content and plasma ALT levels showed signs of improvement. NAC has been clinically used as the mainstay of APAP poisoning. Nevertheless, NAC used individually in this study did not provide satisfactory protection. The combination regimen we tested produced a full protection evidenced by a significant reduction in severity of liver necrosis and the mortality rate reduced to zero. Significant improvement in plasma ALT and liver GSH was also observed. Therefore, this regimen may have a clinical potential and may be the choice of treatment for APAP intoxication in subjects with elevated P450 enzyme activity.

The mechanism of protection by DADS against APAP hepatotoxicity is more likely to be associated with its inhibitory action on hepatic P450 1A1/1A2 activity since *in vitro* enzyme assay demonstrated that DADS is a potent P450 1A1/1A2 inhibitor, although it does not affect induction of this enzyme by BNF[18]. DADS was markedly suppressed, but was unable to completely eliminate the elevated hepatic P450 activity, and the non-suppressed portion of P450 enzyme can still produce an amount of NAPQI sufficient to cause liver necrosis and animal death. On the other hand, NAC does not directly detoxify NAPQI but serves as a precursor of GSH and stimulates hepatic GSH pooling. Hence, effectiveness of measures in enhancing liver detoxifying capability depends upon how potent these prodrugs are and how quickly they can be converted into forms that can be used to detoxify NAPQI. By combination regimen, DADS reduces generation of NAPQI by inhibiting the key enzyme and the NAPQI generated by non-suppressed P450 can be substantially trapped and conjugated by increased GSH due to administration of NAC. Therefore, the combination regimen acts on two steps in the metabolic pathway of APAP to provide the full protection.

Interestingly, the hepatic GSH depletion and plasma ALT elevation in either DADS or NAC group were much less severe than those of BNF group but the death of animals was not avoided. It is likely that adequate maintenance and/or rapid restoration of liver GSH by combination regimen contributes, at least in part, to the full protection. Moreover, plasma ALT level, a commonly used liver damage marker, may not be parallel with the extent of hepatic necrosis and may not truly reflect the severity of intoxication if DADS or NAC had been given. Therefore, plasma ALT level may not be used as a solely reliable parameter to assess the severity of liver damage and to predict the prognosis.

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REFERENCES

1 Miner DJ,Kissinger PT. Evidence for the involvement of N-acetyl-p-quinoneimine in acetaminophen metabolism.*Biochem Pharmacol*,1979;28:3285-3290
2 Hurman AW, Kyle ME, Serroni A, Farber JL. The killing of cultured hepatocytes by N acetyl-p-benzoquinoneimine (NAPQI) as a model of the cytotoxicity of acetaminophen.*Biochem Pharmacol*,1991;41(8):1111-1117
3 Miller MR, Wentz E, Blair JB, Pack D, Hinton DE. Acetaminophen toxicity in cultured trout liver cells.*Experiment Molecul Pathol*, 1993;58:114-126ª¤
4 Roberts WD, Bucci TJ, Benson RW, Warbritton AR, McRae TA, Pumford NR *et al*. Immunohistochemical localization and quantification of the 3-(cystein-S-yl) acetaminophen protein adduct in acetaminophen hepatotoxicity. *Am J Pathol*, 1991;138(2),359-371
5 Birge RB, Bulera SJ, Bartolone JB, Ginsberg GL, Cohen SD, Khairallah EA. The arylation of microsomal membrane proteins by acetaminophen is associated with the release of a 44 kDa acetaminophen-binding mouse liver protein complex into the cytosol.*Toxic Applied Pharmacol*, 1991;109:443-454
6 Holtzman JL. The role of covalent binding to microsomal proteins in the hepatotoxicity of acetaminophen.*Drug Met Rev*, 1995;25:395-451
7 Esterline RL, Ray SD, Ji S. Reversible and irreversible inhibition of hepatic mitochondrial respiration by acetaminophen and its toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). *Biochem Pharmacol*, 1989;38:2387-2390
8 Donnelly PJ, Walker RM, Racz WJ. Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. *Arch Toxicol*,1994;68:110-118
9 Harvison PJ, Guengerich FP, Rashed MS, Nelson SD. Cytochrome P450 isozyme selectivity in the oxidation of acetaminophen. *Chem Res Toxicol*, 1988;1:47-52
10 Lee CA, Thummel KE, Kalhorn TF, Nelson SD, Slattery JT. Inhibition and activation of acetaminophen reactive metabolite formation by caffeine: roles of cytochrome P450 1A1 and 3A2.*Drug Metab Disp*,1991;19:348-353
11 Pezzano M, Richard CH, Lampl E, Pelletier G, Fabre M, Rimaillho A et al. Hepatic and renal toxicity of paracetamol in chronic alcoholic subjects. *La Presse Medicale*,1988;17:21-24
12 Snawder JE, Roe AL, Benson RW, Roberts DW. Loss of CYP2E1 and CYP 1A2 activity as a function of acetaminophen dose: relation to toxicity. *Biochem Biophysic Res Commun*,1994;203(1):532-539
13 Hu JJ, Yoo JS, Lin M, Wang EJ, Yang CS. Protective effects of diallyl sulfide on acetaminophen induced toxicity.*Food Chemical Toxicol*, 1996;34:963-969
14 Zhao C, Zhou YX. Induction of cytochrome P450 1A1 / 1A2 in mouse hepatocytes by β-naphthoflavone: an immunocytochemical study. *Chin J New Gastroenterol*, 1996;4(8):334-335
15 Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brode BB. Acetaminopheninduced hepatic necrosis. I. Role of drug metabolism. *J Parmacol Exp Ther*, 1973;187:185-194
16 Burke MD, Mayer RT. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabol Disposition*,1974;2(6):583-588
17 Patten CJ, Thomas PE, Guy PL, Lee ML, Gonzalez FJ, Guengerich FP *et al*. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem Res Toxicol*, 1993;6:511-518
18 Brady JF, Li D, Ishizaki H, Yang CS. Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. *Cancer Res*, 1988;48:5937-5940

^{99m}Tc labeled HAb18 McAb Fab fragment for radioimmunoimaging in nude mice bearing human hepatocellular carcinoma *

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Subject headings liver neoplasms; carcinoma, hepatocellular; HAb18; autibodies, monoclonal; radioimmunodetection; Fab fragments; ^{99m} Tc; nude mice

Abstract

AIM To establish a method of labeling anti-hepatoma McAb (HAb18) Fab fragment modifier with ^{99m}Tc.

METHODS HAb18 Fab was modified with 2-iminotholane and labeled with ^{99m}Tc by transchelation from ^{99m}Tc GH. Labeling yield, radiochemical purity and immunoreactivity were determined by thin layer chromatography (TLC-SG), paper chromatography (PC), gel chromatography (GC) and cell binding assay, respectively. The nude mice bearing human hepatoma were used for radioimmunoimaging (RII).

RESULTS A radiolabeling yield of 50%-80% was obtained, and immunoreactivity (IR) was 30%-40%. Radioimaging results showed that ^{99m}Tc-HAb18 McAb Fab fragment was concentrated in the tumor 4-8 hours after injection, and the maximum concentration was seen in 12-24 hours, and the T/NT value was 5.18 and 7.48 at 6h and 8h after the injection.

CONCLUSION ^{99m}Tc-HAb18 McAb Fab fragment could be specifically localized in the tumor of nude mice bearing human hepatocellular carcinoma within 24 hours and this method might be effectively used for labeling McAb Fab fragment with 99mTc.

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INTRODUCTION

Many methods have been reported to radiolabel intact MAbs with ^{99m}Tc[1]. Since the long imaging time of intact McAb Hab18 (> 48 hours) is not compatible with the 6h physical $T^{1/2}$ of ^{99m}Tc, it seems preferable to use antibody fragments for ^{99m}Tc labeling, as the fragments have smaller molecular mass, can be cleared more rapidly from the blood, and potentially increase the tumor/non-tumor ratio (T/NT). The method of labeling fragment with ^{99m} Tc is therefore one exploring aspect. In this paper, 2-iminotholane (IT) was used to modify anti-hepatoma McAb HAb18 Fab fragment, and then IT-Fab fragment modifier was labeled with ^{99m}Tc by transchelation from ^{99m}Tc-glucoheptonate (GH). Successful results were obtained when ^{99m}Tc-IT-Fab was injected into the nude mice bearing human hepatoma for radioimmunoimaging (RII).

MATERIALS AND METHODS

Reagents

The anti-hepatoma McAb HAb18 (10g/L, purity >95%) was prepared and supplied by the Hepatoma Targetting Drug Research Laboratory, Department of Pathology, the Fourth Military Medical University. McAb HAb18 belongs to IgG_1 subclass and its affinity coefficient (ka) is 8.17×10.9L/mol. The antigen is about 61kd and has no crossreaction with AFP, CEA, and ferritin. IT is a product of Sigma Chemical Co.; 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB), Fluka Co; papain, Merck Co.; and GH and MDP kit was supplied by the Institute of Jiangsu Nuclear Medicine. TEA-B-1 solution: a mixture of 1.0mol/L TEA and TEA HCl solution (1:1 v/v), pH 8.0; TEA-B2 solution: a mixture of KCl (50mmol/L) and $MgCl_2$ (1mmol/L) with the TEA-B-1 solution; TEA-B-SH solution: 1% 2-mercaptoethanol of TEA-B-2 solution; IT solution: IT (0.25mol/L) dissolved in TEA-B-1 solution.

Human hepatoma nude mice model

Human hepatoma was subcutaneously implanted in the neck or the upper lumber side of NC or BALB/c nude mice. The model was named SMMC-LTNM[2]. The mice were maintained in an aseptic condition and used for experiment when the tumor increased to 0.5 cm-1.4 cm in diameter.

Instrument

Packard A5301D γ-counter produced by Packard Instrument Co.; Diacam/Maxdelta 3000 SPECT system by Siemens Co.; fast protein liquid chromatograpy system (FPLC): Waters 650 with DEAE-sepharose fast flow column; and HPLC: Bio-Rad 5000T.

HAb18 Fab fragment

Prepared by papain digestion and purified by FPLC using DEAE-sepharose fast flow column[3].

Fab fragment modification

Jue's method[4] was used with some modification. Briefly, HAb18 Fab fragment (4g/L) was dialyzed overnight against TEA-B-SH solution, then mixed with IT solution (6mmol/L in concentration) at 0℃ reaction for 20min. The mixture was dialyzed against the TEA-B-2 buffer at room temperature (20℃) for 3h with a buffer change each hour. After that, the mixture was run through sephadex G50 column which was absorbed with bovine serum albumin (BSA), and eluted with 0.05M PBS at a speed of 1 ml/min. The protein fraction was collected under UV-280nm monitoring, concentrated to 4.4g/L, divided into 220μg/vial, and lyophilized for us.

Determination of sulfhydryl groups with DTNB

According to Ellman's method[5], protein samples (0.5ml) were mixed with 2.5ml 0.05mol/L Tris-Hcl buffer (pH 8.4), and 0.025ml 0.01mol/L DTNB solution. Half a mililiter of distilled water instead of protein sample solution was used as blank control. The increase in absorbance at 412nm was followed using a spectrophotometer (Lambda 2S). By means of the formula sulfhydryl group (μmol)= A412/0.5 × 13.6, the number of sulfhydryl group was calculated according to protein concentration (μmol).

^{99m}Tc labeling of IT-Fab fragment

The labeling procedure was as follows: ① 370-555MBq (1ml-1.5ml) of freshly eluted pertechnetate from a ^{99}Mo/^{99m}Tc generator was added into an GH kit (containing 10mg sodium glucoheptonate and 1mg stannous chloride), and was placed 10min, ^{99m}Tc-GH was prepared. ② Lyophilized IT-Fab (220 μg/vial) was dissolved in 100μl 0.1mol/L citrate buffer, pH 6.5, bubbled with N_2. ③ 0.1ml-0.5ml ^{99m}Tc-GH solution was added to the IT-Fab vial and incubated at 37℃ for over 1 hour.

Quality control of ^{99m}Tc-IT-Fab

Radiolabeling yield and radiochemical purity were determined by paper chromatography (PC) using No.1 Xinhua filter paper strips (saline as a solvent, ^{99m}Tc-IT-Fab and $^{99m}TcO_2$ Rf=0-0.1, $^{99m}TcO_4$ Rf= 0.7-0.8. Acetone as a solvent, ^{99m}Tc-GH Rf=0, $^{99m}TcO_4$ Rf = 1); or instant thin layer chromatography (ITLC) using ITLC-SG paper (Gelman Co), (1% BSA pretreated, alcohol: NH_3H_2O:H_2O = 2:1: 5 as a solvent, ^{99m}Tc-IT-Fab and $^{99m}TcO_4$ Rf = 1.0 $^{99m}TcO_2$ Rf = 0) and gel column chromatography (GCC) with sephadex G50 (0.9 cm × 15 cm diameter), 0.05 mol/L PBS, pH 7.4, as an eluent. The protein labeling yield was calculated by routine method. Immunoreactivity assessment: in vitro immunoreactivity of ^{99m}Tc-IT-Fab was evaluated by a conventional live cell assay. Briefly, hepatoma cell suspension was prepared by mincing SMMC-LTNM tumor tissues from the mice model. It was centrifuged and washed with phosphate-buffered saline (0.1 mol/L- PBS); then 10μl diluted ^{99m}Tc-IT-Fab (10×10^3 cpm) and 0.5ml PBS was added, and the mixture was incubated at 37℃ for 2 hours. After incubation, the samples were then centrifuged and the supernatants were removed. The cells were further washed with 1ml PBS solution by centrifugation. Before and after the removal of supernatant, the activity of samples was counted in a γ-counter, and the percentage of binding was calculated.

Animal biodistribution study

^{99m}Tc-IT-Fab 18.5 MBq/0.2ml was intravenously injected into each nude mouse bearing human hepatoma xenografted model by the tail vein, and the mice were sacrificed 5 hours after the injection. The blood and major organs were removed, weighed and counted in the γ-counter for radioactivity, the tumor/blood ratio and the tumor/organ ratios were calculated.

Animal imaging study

^{99m}Tc-IT-Fab 18.5-74 MBq/0.2ml was injected intravenously or intraperitonealy into the tumor-bearing nude mice (n = 6 each in half), and the same dose of ^{99m}Tc-GH was intravenously injected into the nude mice (n = 2) as control. The mice were imaged with a SPECT at 30min, 2 h, 4 h, 6 h, 12 and 22 h after administration. The T/NT ratios were calculated by ROI technique.

RESULTS

Fab fragmant yield was 30%-40%, and single peak was found by HPLC. The molecular mass weight was identified as 45kd by SDS-PAGE. The number of sulfhydryl group per molecule of IT-Fab and Fab fragment was 0.3 and 0, respectively. $^{99m}TcO_2$ was less than 0.5%. The immunoreactivity was 30%-40% by cell binding assay. The biodistribution study showed that the T/NT ratios of tumor to the blood,

the heart, liver, lung, speen, kidney, stomach, intestine, muscle, and brain were 0.89, 4.1, 1.1, 8.0, 5.2, 0.1, 1.6, 4.8, 4.8 and 33.1, respectively. Animal imaging showed that ^{99m}Tc-IT-Fab was rapidly distributed to the whole body when injected i.v. and the radioactivity rapidly appeared in the bladder. The body distribution via intraperitoneal was 10min-30min later than that of the i.v. injection. The tumor was visible with high background at 1h-2h; the accumulation of the tumor was evident with clear kidney imaging 4h-8h after the injection, and a relatively increased tumor localization was seen with low background and decreased kidney outline 12h-22h after injection (Figure 1). The T/NT (on the back muscles) ratios by ROI were 5.18 and 7.48 at 4h and 8h. In control group, ^{99m}Tc-GH rapidly appeared in the blood pool and the whole body, and accumulated rapidly in the bladder. The two kidney imagings were clear, but no tumor accumulation was seen 6h after the injection with two dim kidney outlines and low background. Radioactivity was removed in urine and the bladder image disappeared gradually.

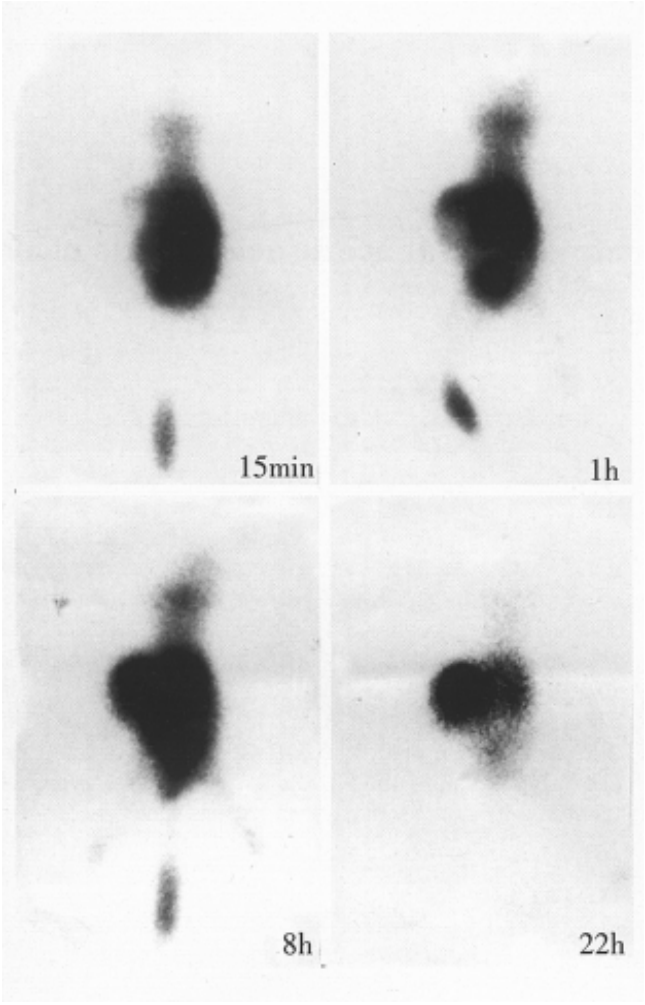

Figure 1 Dynamic imaging of ^{99m}Tc-IT-Fab in nude mice bearing human hepatoma model (posterianterior position).

DISCUSSION

^{99m}Tc labeling McAb F(ab')$_2$ and Fab' fragments have been reported by some authors[1], but Fab fragment was rarely seen. In terms of the clearance, intact IgG was the slowest, F(ab')$_2$ fragment in the middle, and Fab' or Fab fragment the fastest. As for preparation, Fab' fragment was prepared from the reduction of F(ab')$_2$ fragment and then was followed by purifying procedure. The key step was the difficult control of reduction condition. F(ab')$_2$ fragment was prepared and purified from pepsin or papain digestion. Fab fragment was also prepared by papain digestion and then purification, but because of the small number of sulfhydryl group per molecule, Fab fragment was difficult to label with ^{99m}Tc. We used 2-iminothiolane (a chemical compound, MW: 137) to modify Fab fragment, so the number of sulfhydryl group per molecule of IT-Fab was increased from 0 to 0.3. Park *et al*[6] reported that the number of sulfhydryl group per molecular of F(ab')$_2$ was 4.2 and Fab 0.9. The difference might have resulted from the treatment with $SnCl_2$ before the measurement. Rhodes[7] reviewed that the protein to be labeled need to contain free sulfhydryl (-SH) group or reactive monosulfides (-S or -S-metal), and the labeling yield was significantly and positively related to the number of sulfhydryl group. We have modified F(ab')$_2$ with 2-iminothone in the same way (with the number of SH per molecule of 0.7) and labeled with ^{99m}Tc. The labeling yield was 92% by PC, which was higher than that of ^{99m}Tc-IT-Fab (50%-80%). This result proved what was mentioned above. Paik[6] proposed that there were two sites on the IgG molecule, one with a high-affinity and low-capacity and the other with a low-affinity and high-capacity. In our study, 50%-80% of labeling yield was obtained by PC, and 20%-30% by GCC, which suggested that the same phenomenon existed on the McAb fragment. Because of the low-affinity binding, ^{99m}Tc was cleared on the column by Sephadex G_{50}. GH, as a renal function imaging agent, was rapidly excreted from the kidney, and was reported to have some tumor-affinity characteristics[8]. But the present resultsdemonstrated that ^{99m}Tc-GH had no affinity to hepatoma. This proved the specificity of ^{99m}Tc-IT-Fab localized on the tumor. The best tumor imaging was at 12 h-22 h after the administration of ^{99m}Tc-IT-Fab. The background excretion was mainly by kidney route. This was similar to the reported imaging time and excretion route of ^{99m}Tc-Fab'[9]. The biodistribution was mainly in the kidney (32.6% ID/g) and next in the blood, the tumor, liver, and stomach. Our results demontrated that ^{99m}Tc-IT-Fab could bespecifically localized in the tumor of nude

mice bearing human heptocellular carcinoma within 24 hours, and this method could be effectively used for labeling McAb Fab fragment with ^{99m}Tc.

REFERENCES

1 Goldenberg DM, Larson SM. Radioimmunodetection in cancer identification.*J Nucl Med*,1992;33(5):803-810
2 Tao WZ, Kang JS, He DH. Establishment of nude mice bearing human hepatoma model and its pathological characteristics. *J Sec Mili Med Univer* 1989;10(1): 5-8
3 Qiu K, Chen ZN, Liu ZG, Wang Q, He FC, Sui YF et al,Preparation of anti-human hepatoma McAb Hab18 F(ab')$_2$ and Fab fragment by papain degestion.*J Four Mili Med Univ*,1995;16(6):414-418
4 Jue R, Lambert JM, Piece LR, Traut RR. Addition of sulfphydryl groups to escherichia coli ribosomes by protein modification with 2-iminotholane. *Biochemistry*,1978;17(25):5399-5405
5 Ellman GL. Tissue sulfhydryl groups.*Arch Biochem Biophy*, 1959;82(1): 70-77
6 Paik CH, Phan LNB, Hong JJ, Malekeh SS, Susan CH, Richard CR et al. The labeling of high affinity sites of antibodies with ^{99m}Tc. *Int J Nucl Med Biol*, 1985;12(1):3-8
7 Rhodes BA. Direct labeling of proteins with ^{99m}Tc. *Nucl Med Biol*,1991;18 (7):667-675
8 Kuang QF, Guo YZ, Lou B. The preparation of Tc-99m-Glucoheptonate natrium and the results of its animal experiments. *Chin J Nucl Med*, 1986;6(2):73-75
9 Massuger LAG, Claessens RAMJ, Pak KY, Boerman OC, Daddonam PE, Keonders EB *et al*. Tissue distribution of ^{99m}Tc, ^{111}In and ^{123}I-OVTL Fab' fragment in ovarian carcinoma bearing nude mice. *Nucl Med Biol*, 1991;18(1):77-82

Serum deprivation enhances DNA synthesis of human hepatoma SMMC-7721 cells *

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Subject headings liver neoplasms; carcinoma, hepatocellular; DNA, neoplasm/biosynthesis; SMMC-7721; tumor cell, cultured; cell proliferation; growth factors

Abstract

AIM To determine the relationship between serum deprivation or serum levels and cell proliferation of human hepatoma SMMC-7721 cells.

METHODS Human hepatoma SMMC-7721 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS)in 5% CO2 incubator at 37℃ for 24h, and culture media were replaced to serum-free or different serum FCS levels (2.5%, 5%, 10%, 20% and 25%). Six h, 12h, 18h and 24h after the culture, the cells were incorporated [^{3}H]-TdR for 4h. At last [^{3}H]-TdR incorporation was detected with liquid scintillation counting.

RESULTS DNA synthesis of SMMC-7721 cells could be sharply stimulated by short-time (6h) serum deprivation (the cpm value of ^{3}H-TdR incorporation of cells in serum-free was 39.32-fold higher than cells in 25% serum), and the incorporation of 3H-TdR was negatively related to the serum levels. Longer-time serum starvation (12h, 18h and 24h) also greatly stimulated DNA synthesis, although the cpm value of ^{3}H-TdR incroporation was less than that in 6h serum deprivation. Morphology of cells cultured in different serum levels also showed significant difference.

CONCLUSIONS Compared with other cell lines such as BEL7404 and Swiss 3T3, human hepatoma SMMC-7721 cells had different response to the serum deprivation. Short-time serum deprivation could greatly stimulate DNA synthesis of human hepatoma SMMC-7721 cells. Precautions must be given to the changes of serum levels for the detection of growth factors and drugs using SMMC-7721 cells as a model.

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INTRODUCTION

Mammalian cells in culture required serum in order to proliferation[1]. Serum was a mixture of protein and other substances, among which essential hormones and growth factors can support cell proliferation[2]. In order to reduce the effects of growth factors in serum on cells, the procedure of serum deprivation was often involved to study growth regulation on cultured mammalian cells[3-5]. Prolonged serum deprivation induced fibroblastic cells such as Swiss 3T3 to enter a quiescent state (G_0)[6,7]. On the contrary, mouse embryo cells[8] and Hela S-3 cells[9] showed reversal response to serum deprivation and lower serum levels. Human hepatoma SMMC-7721 cells were established in our country[10] and were widely used for detecting the activity of growth factors and drugs. Therefore, it was very important to clarify the effects of serum deprivation and serum levels on the growth and metabolism of SMMC-7721 cells.

MATERIALS AND METHODS

Cell lines and culturing

Human hepatoma SMMC-7721 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in our laboratory. The cells were grown as monolayers in RPMI 1640 medium supplemented with 10%-20% fetal celf serum (FCS) and incubated at 37℃ in the humidified incubator with 5% CO^{2+} 95% air.

Serum deprivation and treatment of serum concentration

Exponent growing cells in flasks were harvested by trypsinization with 0.25% trypsin and suspended in RPMI 1640 medium plus 10% FCS. Cells were plated at 1×10^5 cells/ml in 4 pieces of 24-well plates and incubated at 37℃ in 5% CO^{2+} 95% air for 24 hours. After that, the medium was aspirated and the cells were washed with RPMI 1640 medium. The medium was replaced with RPMI 1640 plus different levels of serum (0%, 2.5%, 5%, 10%, 20% and 25%) in different treatment groups (each group having 4 wells of cells) respectively. The rates of DNA synthesis of cells cultured in different serum levels were detected at interval of 6 hours (6, 12, 18 and 24 hours).

Measurement of DNA synthesis

When cells were cultured in different serum levels for 6, 12, 18 and 24 hours respectively, rates of DNA synthesis were determined by pulse labeling for 4 hours in 74KBq ml^{-1} ^{3}H-TdR. The media were aspirated and the cells were gently rinsed with phosphate buffer saline (PBS), trypsinized properly with 0.25% trypsin, collected on 49 model filter membrane and rinsed with 5% TCA (trichloroacetic acid) and 100% ethanol (three times). The membranes were dried at 80℃ for 30min. The incorporation of ^{3}H-TdR was determined by liquid scintillation counting.

Morphological observation of cells cultured in different serum levels

Before collected for liquid scintillation counting, the cells in different treatment groups were observed and photographed under inverted phase contrast microscope.

Materials RPMI 1640 medium was from Gibco, USA; trypsin was from Sigma;^{3}H-thymidine (^{3}H-TdR) was from the Institute of Atomic Energy of China; 24-well plates were from NUNC; and Triton X100, POPOP, PPO were from Serva.

RESULTS

Effects of serum deprivation and concentration on ^{3}H-TdR incorporation

Serum contained significant amounts of thymidine, which would affect the incorporation of their appropriate exogenous labeled forms. This was beacuse the formulated media such as RPMI 1640 for cell culture did not usually contain unlabeled nucleotides, therefore serum provided the only source other than the labeled form. SMMC-7721 cells were pulse labeled with 3H-TdR for 4 hours at 74KBq ml^{-1} after serum deprivation and treatment of different serum concentrations for different times. The results showed decreased specific activity of the incorporation of ^{3}H-TdR with increased serum concentrations, that was, the incorporation of ^{3}H-TdR had negative relation with serum levels in medium. Cells cultured in serum-free medium for 6 hours after media replacement incorporated ^{3}H-TdR 39.32-fold higher than cells in 25% serum medium (Figure 1A). On the other hand, within 18 hours as the time of serum deprivation going down, the incorporation of ^{3}H-TdR decreased (Figure 1B, C,D). The ratio of 3H-TdR incorporation between cells in serum free and in 25% serum decreased to 3.53-fold (Table 1).

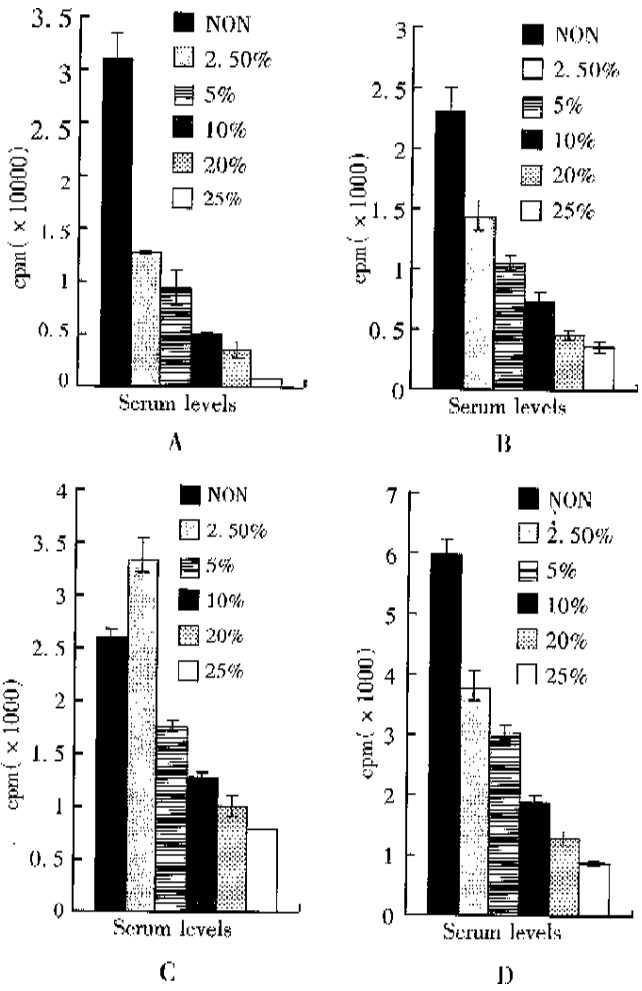

Figure 1 Effects of serum deprivation and serum levels on the incorporation of ^{3}H-TdR.
(A) The cpm value after cells were treated in different serum concentrations for 6 hours; (B) for 12 hours; (C) for 18 hours; (D) for 24 hours.

Table 1 Ratios of the ^{3}H-TdR incorporation among cells cultured in serum-free and different serum levels

Treatments	6 h	12 h	18 h	24 h
SF/2.5% FCS	2.47	1.63	0.778	1.67
SF/5% FCS	3.53	2.26	1.51	1.98
SF/10%FCS	7.34	3.35	2.19	3.27
SF/20% FCS	11.61	5.30	2.72	4.88
SF/25% FCS	39.32	7.21	3.53	7.35

Effects of serum deprivation and serum concentration on cellular morphology

Cells cultured in different serum levels showed significant difference in morphology under inverted phase contrast microscope. Cells cultured in serum-free or lower serum level (2.5%) (Figure 2A) were less well spread and smaller than that in higher serum levels. Cells cultured in 5% serum and more (Figure 2B, C) were epithelial-like and well spread.

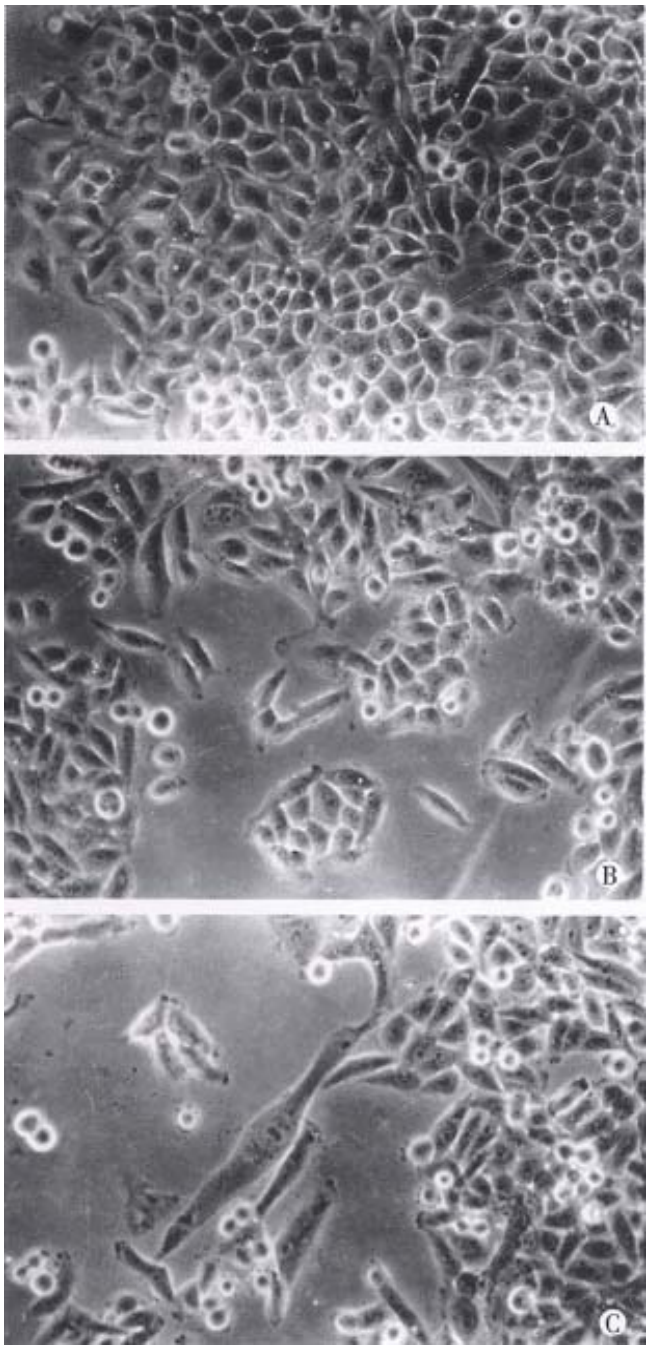

Figure 2A Morphology of human hepatoma SMMC-7721 cells cultured in serum-free medium for 6 hours photographed under phase contrast microscope (200×)
B Morphology of human hepatoma SMMC-7721 cells cultured in 5% serum medium for 6 hours photographed under phase contrast microscope (200×)
C Morphology of human hepatoma SMMC-7721 cells cultured in 25% serum medium for 6 hours photographed under phase contrast microscope (200×)

DISCUSSION

Most mammalian cells were serum dependent and usually passaged in medium containing serum, and they would die if they were cultured in serum-free medium for long time. Therefore, short-time serum-free (serum deprivation) cell culture was one of the main protocols for the study of cell growth and regulation. How cell responses to the serum deprivation has called extensive attention. The results varied greatly even reversal because of the different cells used. Brooks, Larsson[3,4] and Zetterberg[7] indicated that the proliferation of non-transformed fibroblastic cells usually depend on serum or purified growth factors in the tissue culture medium. If the serum concentration was drastically reduced, the cells ceased proliferation and entered a reversible state of quiescence (G_0). Larsson e*t al*[4] reported serum-dependent proliferating 3T3 cells prolonged their intermitotic time by 9-10 hours after exposure to serum-free medium for only 1 hour and a short exposure to serum-free medium was sufficient for cells to leave the cell cycle. Zetterberg[7] showed that in 3T3 cells, in all stages of cell cycle, serum deprivation resulted in inhibition of protein synthesis, but only in postmitotic cells in the first 3 -4 hours of G1 did it produce cell-cycle arrest, a 1-hour exposure to serum-free medium was sufficient to force most G_1 cells into a state of quiescence (G_0). Loo *et al*[8] demonstrated that mouse embryo cells established and maintained in the absence of serum depend on epidermal growth factor for survival and their proliferation was reversibly inhibited by serum or platelet-free plasma. Yin *et al*[9] showed that Hela cells cultured in 10% serum medium incorporated ^{3}H-TdR to 25% of cells cultured in 0.2% serum medium. Xu *et al*[11,12] had demonstrated that human hepatoma BEL-7404 could grow in serum-free medium and there were only less than 2%-3% apoptotic cells after serum starvation for 24 hours. Human hepatoma SMMC-7721 cell were serum-dependent proliferating cell line and widely used in the detection of growth factors and anticancer drugs, therefore it was very important to ascertain the effects of short-time serum deprivation and serum levels on growth and metabolism of SMMC-7721 cells, which would affect the correct assessment of the activity of growth factors and drugs. Our results suggested that short-time serum deprivation (6 hours) could stimulate the synthesis of SMMC-7721 cells and the incorporation of ^{3}H-TdR was negatively related to the serum levels in medium. For longer (12, 18, and 24 hours) exposure to serum^{a2}free medium, the incorporation of ^{3}H-TdR was also negatively related to the serum level, although the ratio of ^{3}H-TdR incorporation of cells in serum^{a2}free medium and in 25% serum medium decreased from 39.32-fold (6 hours) to 3.53-fold (18 hours). These results were reversal to that with 3T3 cells, which could easily be overlooked, or even mistakenly attributed to the activity of growth factors and drugs. The results might be due to two main reasons, one was that DNA synthesis of SMMC-7721 cells cultured in serum-free medium was

inhibited by the lack of thymidine in RPMI 1640 medium and the cells were accumulated to the G_1/S. Once ^{3}H-TdR was added to the medium, the cells began to enter S phase quasisynchronously and start DNA synthesis, the other reason was that most of malignant cells appeared less dependent on serum and could secrete some growth factors and stimulate themselves by feedback mechanism. The differences of cell morphology in serum-free and different serum levels were mainly induced by fibronectin and fetuin in serum[13], which promoted cell attachment and spread on the surfaces of culture plates. So necessary precautions must be given to the serum level changes in the medium in detecting growth factors and drugs with human hepatoma SMMC-7721 cells, otherwise false conclusion might be implied.

REFERENCES

1 Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science*, 1955; 122(3168):501-504
2 Hayashi I, Sato G. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature*, 1976;259(5539):132-134
3 Brooks RF. Regulation of the fibroblast cell cycle by serum. *Nature*, 1976;260 (5548):248-250
4 Larsson O, Dafgard E, Engstrom E, Zetterberg A. Immediate effects of serum depletion on dissociation between growth in size and cell division in proliferating 3T3 cells.*J Cell Physiol*, 1986;127(2):267-273
5 Zaitsu H, Kimura G. Serum-dependent regulation of proliferation of cultured rat fibroblasts in G1 and G2 phases. *Exp Cell Res*, 1988;174(1):146-155
6 Campisi J, Morreo G, Pardee AB. Kinetics of G1 transit following brief starvation for serum factors.*Exp Cell Res*, 1984;152(2):459-466
7 Zetterberg A, Laesson O. Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells.*Proc Natl Acad Sci USA*,1985;82 (16):5365-5369
8 Loo DT, Fuquary JI, Rawson CL, Barnes DW. Extended culture of mouse embryo cells without senescence: inhibition by serum. *Science*, 1987;236(4798): 200-202
9 Yin Z, Wheatley DN. Sensitivity of 3T3 cells to low serum concentration and the associated problems of serum withdrawal. *Cell Biology*, 1994;18(1):39-46
10 Tong R, Zhou R, Lu F, Tao W. The establishment and primary biological characteristics of human hepatoma SMMC-7721 cell line.*J 2nd Milit Med Univ*, 1980; 1(1):5-9
11 Xu Y, Jiang W, Peng S, Chen Y. Antisense EGFR sequence reverses the growth properties of human liver carcinoma cell line BEL-7404 in vitro. Cell Res,1993;3 (1):75-83
12 Fu T, Liu H, Liu F, Gu J, Jiang W, Xu Y. Antisense EGFR sequence enhances apoptosis in a human hepatoma cell line BEL-7404.*Cell Res*, 1996;6(2): 145-153
13 Freshney RI. Culture of animal cells. New York: *Alan R Liss Inc*, 1983:74-77

Codon 249 mutations of p53 gene in development of hepatocellular carcinoma

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Subject headings liver neoplasms; carcinoma, hepatocellular; p53 gene; mutation; RNA, messenger; LOH; codon 249; immunohistochemistry; polymerase chain veaction

Abstract

AIM To investigate the mechanisms of codon 249 mutation of p53 gene in the formation of hepatocellular carcinoma (HCC).

METHODS Codon 249 mutation accompanied by loss of heterozygosity (LOH) and its effect on translation and transcription were studied using SSCP, IHC and RT-PCR/slot hybridization.

RESULTS Codon 249 mutations were detected in 32.9%, LOH detected in 68.4% among the HCC patients. Mutations of condon 249 were accompanied by LOH in 90%. The positive rates of p53 protein and mRNA were 91.3% and 95.7%, in mutational group, both were significantly higher than those in the non-mutational group (91.3% *vs* 19.1% and 95.7% vs 40.4%, respectively, both $P < 0.01$). The translation of p53 gene was strongly related to its transcription by correlation analysis ($r = 0.8208$).

CONCLUSIONS LOH might play an important role in hepatocarcinogenesis of codon249 mutation, which could increase both transcription and translation of p53 gene. The increased expression of p53 protein mainly depend on the increased transcription of p53 gene.

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INTRODUCTION

HCC is one of the most common human tumors in certain areas of Africa and Asia including southern China. However, the mechanisms, especially the molecular mechanisms of hepatocarcinogenesis are still not well understood. Since condon 249 mutations of p53 gene were observed in 45%-50% of HCCs from southern Africa and Qidong, China, it has been believed that mutations of codon 249 plays an important role in hepatocarcinogenesis[1-3]. But now mutation causes HCC, is still unclear. Mutant p53 proteins with much longer half-life are often overexpressed in HCC. They can inactivate the wild proteins through dominant negative in vitro[4-7]. Losses of heterozygosity are often observed in HCC. They are favorable for mutations to enact[8-10]. Nevertheless, the effect of codon 249 mutations on transcription or translation of p53 gene and the relationship between these mutations and LOH are still unclear. Therefore, in this study, we investigated these questions using surgical specimens of HCC from southern China where the incidence of HCC is moderate to high.

MATERIALS AND METHODS

Materials

Specimen Seventy surgical specimens of HCC were collected from the First Affiliated Hospital and Cancer Hospital, Sun Yat-Sen University of Medical Sciences. Cancerous and pericancerous tissues were separated by pathologists.

Reagents Restriction enzyme Hae III was purchased from GIBco BRL. p53 protein monoclonal antibody (DO-7) and LSAB system were purchased from DAKO company. Dig detection kit was from B. M. Company.

Methods

Examination of codon 249 mutation using RFLP analysis Exon 7 was amplified using primers 5'-GGCGA CAGAG CGAG ATTCCA-3' (sense) and 5'-GGGTC AGCGG CAAGC AGAGG-3' (antisense). Twenty μl amplified DNA product (286bp) was digested with 2.5U- restriction enzyme Hae III. DNA fragments with and without digestion were analyzed in 2% agarose gel.

Examination of p53 gene LOH using SSCP analysis

Exon 3-4 was amplified using primers 5'-AAATT CATGG GACTGACTTT-3' (sense) and 5'-AATGC AGGGG GATAC GGCCA GC-3' (anti-sense). PCR product was precipitated at -20℃ for 1 hour by adding 2.5vol of alcohol and 1/10vol of 4M sodium acetate. Pellets were resuspended in 10μl Formamide dye mixture (95% formamide, 20mmol/L EDTA, 0.05% bromphenol blue). Samples were heated at 95℃ for 5 minutes, chilled on ice and immediately loaded (5μl) on 6% acrylamide/TBE- gel. fGels were run at 40W for 4 hours at room temperature. Silver-staining was used to visualize the bands. LOH of p53 gene was ensured by comparing the band pattern from cancerous tissue and pericancerous tissue of the same patient.

Detection of p53 proteins using immunohistochemistry p53 proteins in tissues were detected using immunohistochemistry (IHC). Semi-quantification was carried out in 5 groups of patients divided according to the percentage of positive cells (<1%, 1%-5%, 5%-25%, 26%-50% and >50%).

Detection of p53 mRNA using RT-PCR Total cell RNA was extracted using Chomczynski's method. DNA fragments of 555bp were amplified using RT-PCR with primers 5'-TACTC CCCTG CCCTC AA-3'(sense) and 5'-GTTGG GCAGT GCTCG CT-3' (anti-sense)[11]. RT-PCR products were transferred to nitrocellulose membrane. p53 mRNA was detected by hybridization with the Dig-probes which were labeled with PCR, and then visualize the dots with Dig detection kit. The degree of dot color was classified into 5 grades.

RESULTS

Mutation of codon 249

Exon 7 of cancerous and pericancerous tissues from 70 cases of HCC were all amplified satisfactorily. The number of base pairs of PCR product was as the same as designed. Mutations of codon 249 were detected in 23 of these cases, with a positive rate of 32.9%. One of them showed partial mutation, which was confirmed to be an allele mutation through SSCP analysis. Typical results are shown in Figure 1.

LOH of p53 gene

Exon 4 of p53 gene has a heterogeneous location (CGC/CCC) at condon 72. The number, however, of band pattern in this study was more than 4. It might suggest that there were two or more heterogeneous locations in DNA fragment amplified from exon 3 to 4. The increased heterogeneous locations were favorable to LOH analysis. Heterozygous form was found in 57 of 70 (81.4%) cases of HCC and LOH was detected in 39 of the 57 (68.4%) cases which carried heterozygous alleles. Heterozygous alleles were found in 20 of 23 cases with mutant codon 249, 18 of which (90%) were mutant allele accompanied by LOH. Typical results are shown in Figure 2.

Expression of p53 protein

p53 proteins were detected only in cancerous tissues of HCC. Positive cells 7% were found in 30 cases, with a rate of 42.9%. Typical positive stain is shown in Figure 3. The relationship between the expression of p53 proteins and codon 249 mutations is shown in Table 1.

Relationship between expression of p53 protein and p53 mRNA

The relationship between the expression of p53 proteins and the expression of p53 mRNA among 23 cases carrying codon 249 mutation is shown in Figure 5. Grade correlation analysis demonstrated that they were strongly correlated (correlation coefficient $r = 0.8208$).

Expression of p53 mRNA

The results of p53 mRNA detection are shown in Figure 4. p53 mRNA positive rate was 58.6%. The relationship with codon 249 mutation is shown in Table 1.

Table 1 The relation between codon 249 mutation and expression of p53 proteins and p53 mRNA

Mutations of codon 249	Cases p53	proteins	p53 mRNA
+	23	21[b]	22[d]
-	47	9	19

[b]$P<0.01$, *vs* codon 249 mutation negative group, [d]$P<0.01$, *vs* codon 249 mutation negative group.

DISCUSSION

Tumor suppressor p53 gene often shows mutational hotspot of codon 249 in HCC, i.e., selective G to T mutation usually occurs in the third base of codon 249. This unique mutation has been reported to be involved in the carcinogenesis, differentiation and metastasis of HCC[1-6]. How this mutation causes HCC, however, is unclear. It is believed that some codon mutations of p53 gene can prolong the half-life of p53 proteins. Thus, the mutant p53 proteins often had overexpression in HCC, and inactivated its antitumor effect in combination with wild p53 proteins[4-7]. However, the effect of mutation on the transcription and translation of p53 gene is unclear. This study showed that the p53 mRNA expression in mutational group was significantly higher than that of non-mutational group. The expression of p53 proteins was strongly correlated to the expression of p53 mRNA. These results suggested that codon 249 mutation could increase p53 mRNA expression, and increased mRNA was an important factor in the overexpression of mutant p53 proteins.

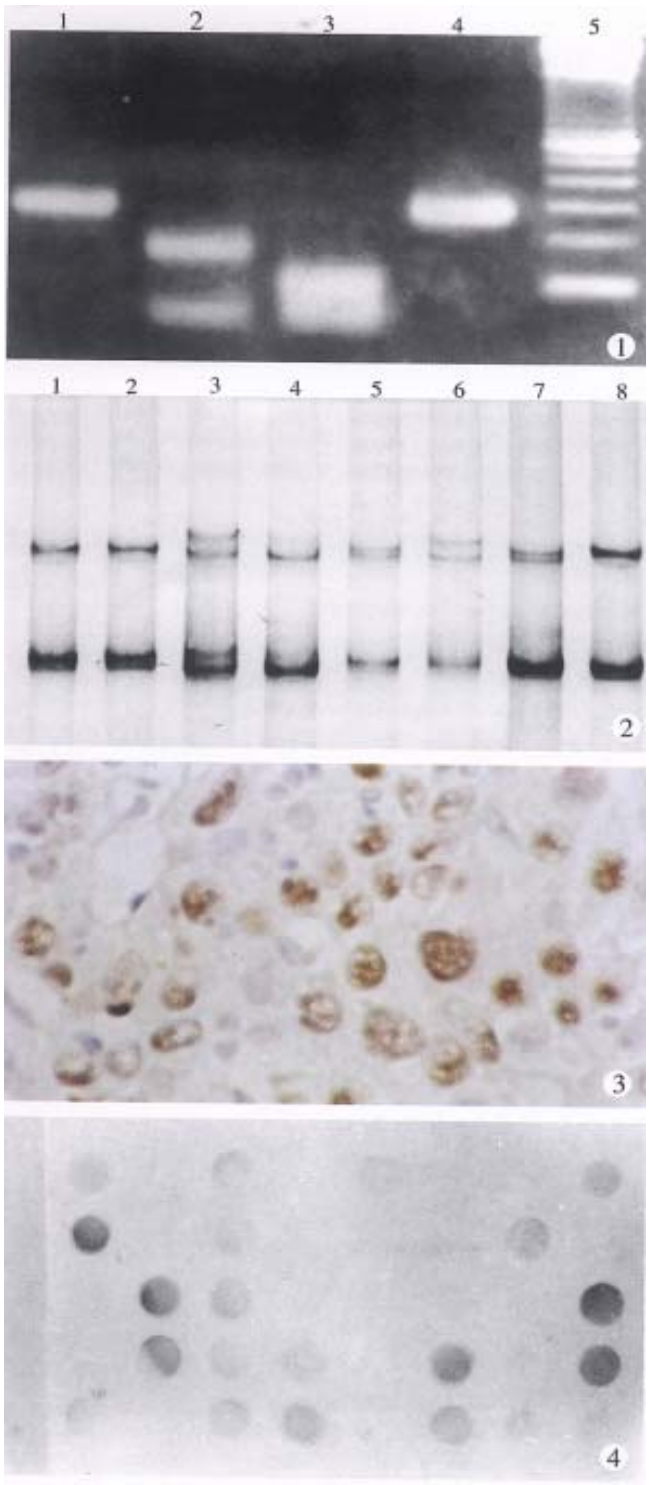

Figure 1 Examination of codon 249 mutation using RFLP analysis. Lane 1 and lane 2 were PCR products from cancerous tissues without and with digestion of restriction enzyme Hae III. Lane 3 and lane 4 were products from pericancerous tissues with and without digestion of restriction enzyme Hae III. Lane 5 was marker of 100bp ladder from GIBco BRL.

Figure 2 The PCR/SSCP detection of LOH, silver-staining. Lane 1 and lane 2 were products from patient 1 who was homozygote. Lane 3 to 8 were products from patients 2 to 4. They were all heterozygote. Patients 2 and 4 were LOH positive. Patient 3 has mutation of one allele.

Figure 3 Expression of p53 proteins in HCC tissue using IHC (×400).

Figure 4 p53 mRNA detected using RT[a2]PCR/slot hybridization.

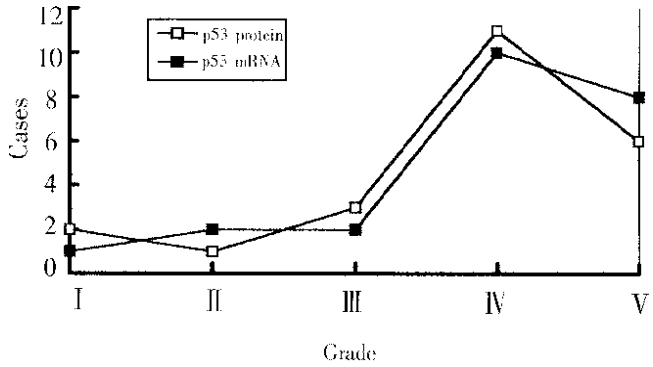

Figure 5 The relation between the expression of p53 proteins and that of p53 mRNA.

When one allele mutated, it was difficult to amass enough cells to allow the other allele to mutate[7]. Therefore, it is important to study whether LOH exists in the other allele. Our results showed that 90% codon 249 mutations were accompanied by LOH of the other allele, suggesting that LOH of p53 gene was also important in hepatocarcinogenesis as mutations. LOH might be favorable for mutations to enact.

In conclusion, HCC will be prevented if we take some measures to avoid recurrent hepatic damage, and reduce the occurrence of LOH, and the clinical outcome of HCC will be much better if the transcription of p53 mRNA can be suppressed by chemical or gene therapy.

REFERENCES

1 Hsu IC, Metcalf RA, Sun T, Welsh J, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*, 1991;350(6317): 427-428
2 Peng XM, Peng WW, Yao JL, Zhou YP. The relationship between hepatitis c and bvirus infection and aberration of p53 gene. *Chin J Hepatol*, 1997;5(2): 124-125
3 Soini Y, Chia SC, Bennett WP, Groopman JD, Wang JS, DeBenedetti VM *et al*. An aflatoxin-associated mutational hotspot at codon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. *Carcinogenesis*, 1996;17(5):1007-1012
4 Hayashi H, Sugio K, Matsumata T, Adachi E, Takenaka K, Sugimachi K. The clinical significance of p53 gene mutation in hepatocellular carcinomas from Japan. *Hepatology*,1995;22(6):1702-1707
5 Qin L, Tang Z, Liu K. The relation between p53 mutations and tumor invasiveness of human hepatocellular carcinoma. *Chin J Cancer*, 1995;17(6):405-408
6 Hollstein M, Sidransky D, Vogelistein B, Harris C. p53 mutations in human cancers.*Science*,1991, 253(5015):49-53
7 Ng IO, Lai EC, Chan AS, So MK. Overexpression of p53 in hepatocellular carcinomas: a clinicopathological and prognostic correlation.*J Gastroenterol Hepatol*,1995;10(3):250-255
8 Rogler CE, Chisari FV. Cellular and molecular mechanisms of hepatocarcinogenesis.*Sem Liver Dis*,1992;12(3):265-278
9 Yumoto Y, Hanafusa T, Hada H, Morita T, Ooguchi S, Shinj N *et al*. Loss of heterozygosity and analysis of mutation of p53 in hepatocellular carcinoma.*J Gastroenterol Hepatol*,1995;10(2):179-185
10 Lasko D, Cavence W. Loss of constitutional heterozygosity in human cancer. *Annu Rev Genet*,1991;25:281-314
11 Shieh YSC, Nguyen C, Vocal MV, Chu HW. Tumor-suppressor p53 gene in hepatitis C and B virus associated human hepatocellular carcinoma. *Int J Cancer*, 1993;54(4):558-562

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Endotoxins enhance hepatocarcinogenesis induced by oral intake of thioacetamide in rats *

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Subject headings liver neoplasms; carcinoma, hepatocellular; endotoxins; thioacetamide; glutamyl transpeptidase/metabolism; flow cytometry; DNA, neoplasm; rats, Wistar

Abstract

AIM To clarify whether endotoxin is of pathogenic importance for hepatocarcinogenesis,or the increased cancer risk results solely from thecirrhotic process.

METHODS The rat model of hepatoma was treated by the intake of 0.03% thioacetamide in drinking water for six months. During induction of hepatoma, rats were additionally treated with splenectomy and/or lipopolysaccharide administration. The liver nuclear DNA index and proliferation index were quantitatively analyzed by flow cytometry. Hepatic histology was examined with light and electron microscopes. Plasmic endotoxin concentration and γ-glutamyl transpeptidase activity were measured, and hepatoma incidence was recorded.

RESULTS Thioacetamide induced cirrhosis and hepatoma in Wistar rats with histology or regenerative nodule, fibrosis and neoplastic foci were quite similar to the pathogenic process of human cirrhosis leading to hepatoma. In comparison with TAA controls (DNA index: 1.15±0.21), exo-endotoxin increased the DNA index by 7.8% (1.24±0.25, $P<0.02$) and hepatoma rate by 16.7. Splenectomy-induced enteric endotoxemia increased the DNA index by 25% (1.44±0.15, $P<0.01$) and hepatoma rate by 33%. A summation of the effects of these two factors increased the DNA index by 36% (P£¼0.01)and hepatoma incidence by 50%, moreover, the level of endotoxemia showed a close relation with DNA index ($r = 0.96$, $P<0.01$), as well as with the occurrence rate of hepatoma ($r = 0.00$, $P<0.01$). Histological findings further verified such alterations.

CONCLUSION Lipopolysaccharide administration and/or splenectomy-induced enterogenic endotoxemia may enhance rat hepatocarcinogenesis induced by oral intake of thioacetamide.

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INTRODUCTION

Endotoxemia is often associated with hepatic injury and cirrhosis[1]. Patients with hepatic cirrhosis or hepatocarcinoma often present a 3 to 8-fold high systemic endotoxemia above normal[2]. If cirrhosis is established, there is a 200-fold increased risk for development of hepatocarcinoma[3]. It is unknown whether endotoxin is of importance in hepatocarcinogenesis. Recognized etiologic or predisposing factors include chronic liver disease caused by hepatitis B, C and D; hepatotoxins such as aflatoxin and alcohol; and cirrhosis of any cause[4]. The pathogenetic mechanisms involved in human hepatocarcinogenesis remain largely unsolved.

Numerous animal models of cirrhosis have been developed[5,6]. Continuous administration of thioacetarnide (TAA) in drinking water to rats produced cirrhosis in 4 months and hepatocarcinoma in 6 months[7]. The histology of the TAA model was more similar to human cirrhosis than that of the CCl_4-treated rats[8]. Wistar rats were used in the TAA model.

Using a TAA-induced rat model of cirrhosis leading to hepatocarcinoma, we tried to prove the hypothesis that lipopolysaccharide (LPS) administration and/or splenectomy-induced enterogenic endotoxemia may play an important role in the pathogenesis of liver tumors. The present study was designed to investigate the histologic changes of hepatocarcinoma in the TAA models treated with LPS and/or splenectomy; DNA aneuploidy and incidence of hepatocarcinoma in various groups; and the relationship between endotoxemia and hepatocarcinogenesis.

MATERIALS AND METHODS

Animals and treatment

Female Wistar rats (Experimental Animals Center of Shanxi Medical University) weighing 125 g ± 9 g, were housed in wire-bottom cages under a 12-hour light/dark cycle and fed with a balanced pellet diet

ad libitum. Animals were randomly assigned to five groups: sham group (n = 5) as an untreated control receiving tap water ad libitum; TAA control group (n = 6), given 0.03% w/v thioacetamide (TAA, purity>99%, Shanghai Central Chemical Factory) in drinking water; TAA+ST group (n = 6) splenectomized (ST) a week before the experiment; during the last two months, 0.8mg% lipopolysaccharide (LPS, from -Escherichia coli-stereotype 055:B5, Sigma) added to drinking water containing 0.03% TAA for both TAA+LPS group (n = 6); and TAA +ST+LPS group (n = 6). Without splenectomy, animals underwent midline laparotomy and spleen manipulation as a sham surgery. All operations were performed under light ether anesthesia and sterile conditions. At the termination of a 6-month course, rats were anesthetized with ethyl ether and blood from abdominal aorta was collected in heperinized tubes for measurement of plasmic endotoxin concentration and glutamyl transpeptidase (GGT) activity.

Analysis of endotoxin and γ-glutamyl transpeptidase activity in plasma

Endotoxin in plasma was determined by means of a chromogenic limulus amebocyte lysate (LAL) assay[9]. Limulus kit (Shanghai Yihua Clinical Medical Biotech Co.) was used for endotoxin-specific quantitative measurement in this study. Syringes, needles and glasswares were made pyrogen-free with dry heat (200℃ for 4 hours). The plasma was pretreated with perchloric acid (PCA) for removal of the possible inhibitors to LAL assay. Briefly. 0.1ml of 0.18mol/L NaOH was added to a 0.1ml of test plasma and incubated at 37℃ for 5min. Then 0.1ml of 0.32mol/L PCA was added for an additional 10min incubation. The precipitate formed was dissolved by addition of 0.2ml of 0.18mol/L NaOH with vigorous mixing. The solution was centrifuged at 2000rpm for 10min. Then 0.05ml of the supernatant diluted with 0.05ml of 0.4mol/L Tris-HCl (pH 8.0) was added to 0.1ml of limulus amebocyte lysate (LAL) in pyrogen-free cuvette, and incubated in a thermostatic bath at 37℃ for exactly 30min. The synthetic chromogenic substrate 0.05ml, *n*-tert-butoxycarbonyl-*L*-leucyl-*L*-glycyl-*L*-arginine-*p*-nitroaniline (Boc-Leu-Gly-Arg-PNA) was added to the lysate and incubated for 5min. The reaction was stopped with 0.5% sodium nitrite. The amount of PNA released from the substrate was detected after diazo-coupling by adding 0.5ml of 0.05% ammonium sulfate and 0.5ml of 0.05% *n-l*-naphthylethylenediamine dihydrochloride. Absorbance was read at 545nm on 721A spectrophotometer, using distilled water as the 0 absorbance value. A standard curve was plotted with the known endotoxin concentration for each run. Simultaneously, saline replacing plasma was as reagent control.

γ- glutamyl transpeptidase (GGT) activity in plasma was detected according to the method as described by Xu KC[10]. In brief, 0.1ml of plasma was added to a 0.5ml of 10mmol/Lsubstrate, γ-L-glutamyl-α-naphthytamide (Shanghai Third Reagent Factory), and incubated at 37℃ for 120min. The amount of α-naphthylamide released from the substrate was detected after adding 10ml of 0.2% sulfanilicacid and 0.004% sodium nitrite. The absorbance was read at 500nm on a 721A spectrophotometer, using sample control of adding plasma after 120min incubation as the 0 absorbance value. A standard curve was plotted with the known α-naphthytamide concentration for each run.

Light and electron microscopy

One half of the left lobe of the liver was resected, fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (HE) for light microscopy. For electron microscopic study, the selected areas with hepatocarcinoma were cut into small pieces and fixed in 2% glutaraldehyde for 2 hours. After post-fixation in 1% osmium tetroxide, the fragments were dehydrated in a graded alcohol series and embedded in Polybed. They were trimmed into ultra-thin section and stained with uranyl acetate and lead citrate, and observed under an electron microscope at 100KV.

Flow cytometry

According to Zuo LF[11], with slight modification. The specimens for flow cytometry were prepared immediately after removal of liver. About 2g tissue was mechanically minced in 5ml of 0.05% collagenase (type Ⅳ, Sigma) and then through a 200 mesh filter. After washing with 0.1mol/L phosphate buffer solution (PBS), the well-dispersed hepatocytes were resuspended and fixed in 70% ethanol overnight, the cells were stained with propidium iodide reagents (propidium iodide 50mg/L, RNase 20mg/L, and Triton-X-100 1%) at 4℃ for 30min. Chicken erythrocytes were used as internal controls. Flow cytometry was performed on a Fluorescence Activated Cell Sorter (FACS) 420 cytometer (USA). Data were analyzed on a compatible computer with HP-300 consort 30. A total of 10^4 cells were examined for each sample, DNA index (DI) expressed as a relative DNA content, wascalculated by dividing mean channels of G0/1 peak in the treated rat by that of sham group. The coefficient of variation (CV) of G0/1 peak of the DNA ploid population was expressed by normalized standard deviation (CV=standard deviation/mean peak position×100). CV was always less than 5%. For the

DNA ploid criteria, it was classified as DNA-dipoid if DI=1.0±2CV, and DNA-aneuploid if DI≠1.0 ±2CV. Cell cycle parameters were analyzed by DNA cell cycle analysis software Sum of Broadened Rectangles Model, and the cell proliferation index (PI) was expressed as the percentage of cells in S plus G2M phases.

Statistical analysis

All results were expressed as x ± s. Data were analyzed by Student's *t* test and multiple regression. *P* values <0.05 were considered statistically significant.

RESULTS

The oral intake of TAA in drinking water induced cirrhosis and, in some rats with hepatocarcinoma with null mortality. TAA-treated animals showed a progressive decline in body weight, particularly in the first month (data not shown). On the contrary, the ratio of liver weight to body weight were obviously higher in all TAA treated animals ($P<0.01$), compared with the sham group. The hepatocyte proliferation index was fairly high in the TAA+ LPS or TAA+ST groups, and the highest in the TAA+ST+LPS group ($P<0.05$) compared with the TAA-model control (Table 1).

Table 1 Hepatocyte proliferation and liver weights

Groups	*n*	PI	Ratio of liver weight to 100g body weight
Sham	5	0.25±0.032	4.32±2.6
TAA control	6	0.33±0.047	8.38±2.0[b]
TAA+LPS	6	0.35±0.045	7.35±1.3[b]
TAA+ST	6	0.35±0.077	7.40±1.5[b]
TAA+ST+LPS	6	0.45±0.039[a]	7.28±1.5[b]

[a]$P<0.05$, *vs* TAA control, TAA+LPS and TAA+ST groups respectively. [b]$P<0.01$, *vs* sham group. PI, proliferation index. Rats were treated as described in Materials and Methods.

Endotoxemia level and GGT activity in systemic circulation

The TAA-treated rats with splenectomy or administration of 0.8mg% LPS (approximately corresponding to 50 μg/100 g body weight daily) in drinking water during the last two months of the study showed increased endotoxemia level ($P < 0.05$), compared with the sham group. The blood endotoxin level in the TAA+ST+LPS group further mounted up ($P < 0.05$), compared with the sham group or the TAA control group. However GGT activity was markedly increased in the blood of the TAA-treated rats with splenectomy plus LPS administration ($P < 0.05$), compared with solely TAA-treated group (Table 2).

Table 2 Endotoxemia levels and GGT activities in plasma

Groups	*n*	Endotoxin (EU/100ml)	GGT (U/100ml)
Sham	5	95±15	17±7
TAA control	6	108±12	226±33[d]
TAA+LPS	6	118±12[d]	236±44[d]
TAA+ST	6	130±31[d]	266±127[d]
TAA+ST+LPS	6	130±18[ad]	442±99[abcd]

[a]$P<0.05$ *vs* TAA control; [b]$P<0.05$, *vs* TAA+LPS group; [c]$P<0.05$, *vs* TAA+ST group; [d]$P<0.05$, *vs* sham group. Rats were treated as described in Materials and Methods.

DNA index and hepatoma rate

The livers of rats in the TAA control group showed many superficial yellowish macronodules and one case of visible hepatocarcinoma out of six rats. With LPS administration and/or splenectomy, numerous micronodules appeared in livers and more cases of noticeable tumors. Moreover much more hepatocarcinomas were observed under the light microscope. DNA index as an indicator of the severity of malignant lesions were stepped up in the TAA models additionally treated with LPS and/or splenectomy. Therefore, they have significant differences between groups ($P<0.02$), particularly in the splenecotomized TAA-treated rats with oral LPS administration (Table 3).

Table 3 DNA ploidy and incidence of hepatoma in various groups

Groups	*n*	DNA index	Rate of DNA aneuploid%	Incidence of hepatoma			
				Visible		Under microscopy	
				(%)	*n*	(%)	*n*
Sham	5	1.0	0	0	(0/5)	0	(0/5)
TAA control	6	1.15±0.21	50	17	(1/6)	50	(3/6)
TAA+LPS	6	1.24±0.25[a]	50	33	(2/6)	50	(3/6)
TAA+ST	6	1.44±0.15[ab]	100	50	(3/6)	67	(4/6)
TAA+ST+LPS	6	1.56±0.07[abc]	100	67	(4/6)	67	(4/6)

[a]$P<0.02$, *vs* TAA control group; [b]$P<0.02$, *vs* TAA + LPS group; [c]$P<0.02$, *vs* TAA + ST group. DNA index=mean channels of G0/1 peak in the treated rat ÷ mean channels of G0/1 peaks in the normal controls. 10^4 cells of a rat liver were examined by flow cytometry. Rats were treated as described in Materials and Methods.

Histology

Under the light microscope, no noticeable changes were observed in untreated control rats (Figure 1). The livers of the TAA models with or without LPS and/or splenectomy had numerous micronodules of various sizes in all lobes. The nodules were separated by markedly proliferated collagen fiber, forming pseudolobules. Proliferation of spindle cells, probably fibroblasts, was recognized around such hyperplastic nodules (Figure 2). In the transforming

hepatocytes, atypical hyperplasia with nuclear heterochromatism and basophilic cytoplasm were seen mainly in perivenular and in periportal areas. Nuclear atypism was also seen in the hepatocytes. Moreover, disarrangement of the hepatic cords became apparent and formed considerable pseudoglandular (acinar) structures which were transformed from original liver parenchymal cells (Figure 3). Highly malignant lesions were observed in the TAA +ST+LPS group, in which atypism was more apparent and formed a "mucoid lake" with a float of small atypical cells (Figure 4).

Electron microscopic investigation clearly showed that the disarrangement of the altered hepatocytes and nuclear atypism were very apparent, together with less cytoplasm containing very few organelles (Figure 5). Collagen fibrils were markedly present around the neoplastic cells (Figure 6).

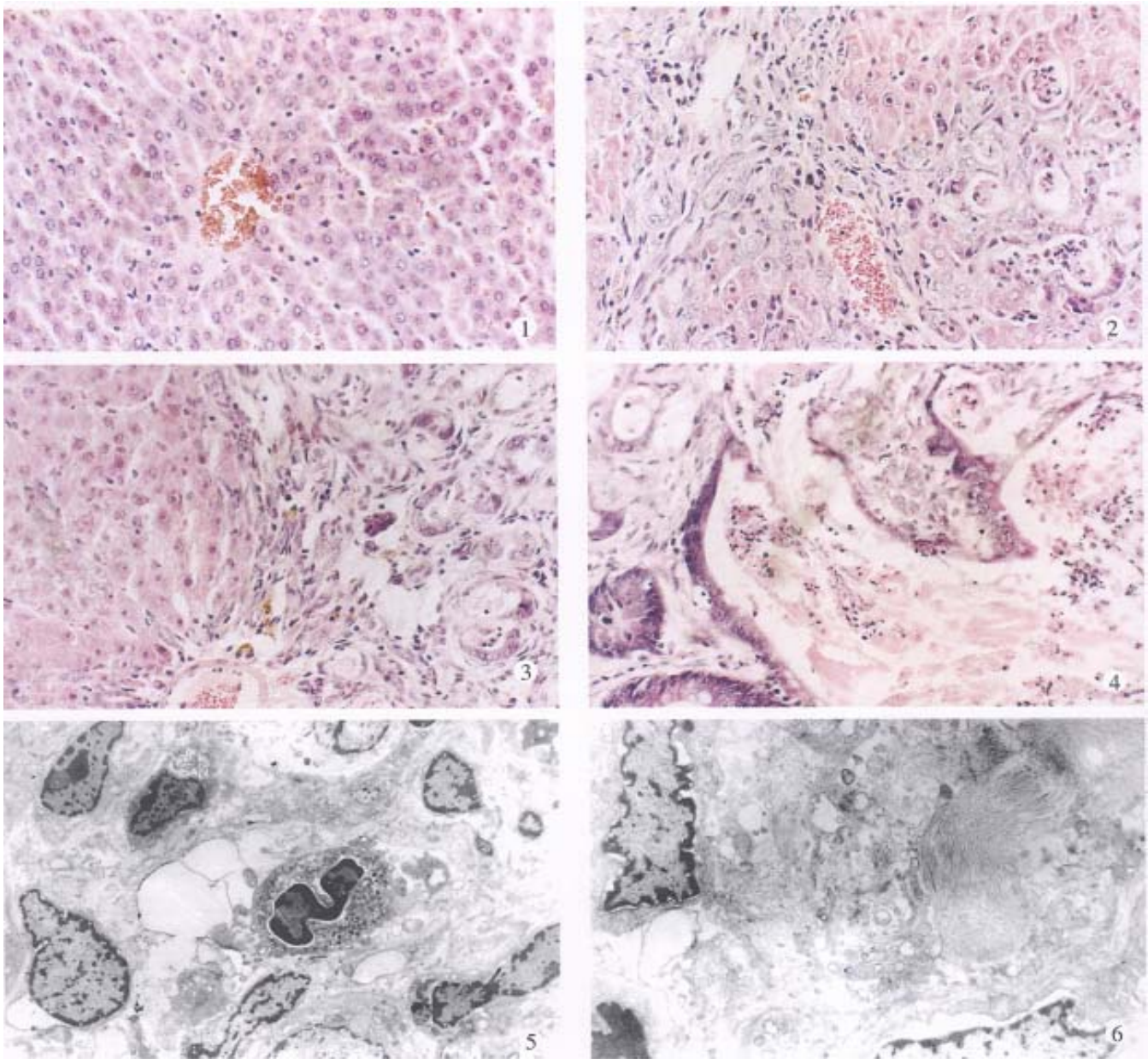

Figure 1 Section of liver from an untreated control (sham) rat. HE×20.

Figure 2 Section of liver from a TAA control rat treated as described in Materials and Methods. The nodules show the transforming hepatocytes and the hepatocarcinoma. The spindle fibroblasts are recognized around such hyperplastic nodules. HE×20.

Figure 3 Section of liver from a rat in TAA+ST group. Pseudoglandular structures and spindle cells are observed in perivenular areas. HE×20.

Figure 4 Section of liver from a rat in TAA+ST+LPS group. The disorganized and atypical hepatocytes formed a "mucoid lake" with floating of small cells. HE×20.

Figure 5 Ultrastructure of hepatocellular carcinoma from a rat in TAA+ST+LPS group. Uranyl acetate and lead citrate staining ×400.

Figure 6 Ultrastructure of cirrhosis from a rat in TAA+ST+LPS group. The bundles of collagen fibrils were present with atypical hepatocytes. Uranyl acetate and lead citrate staining ×800.

DISCUSSION

TAA-induced cirrhosis and hepatocarcinoma has been observed in Wistar rats. Theresults of the present study demonstrated for the first time that endotoxin may play an important role in the development of hepatocarcinoma in rats induced by continuous administration of 0.03% TAA in drinking water.

TAA is hepatotoxic owing to effects on DNA, RNA, protein synthesis and GGT activity, through which it induces cirrhosis and hepatocarcinoma[1,8]. After 6 months of chronic TAA administration, the liver exhibited an external appearance of cirrhosis characterized by the presence of numerous yellowish nodules and whitish hepatocarcinoma. The microscopic study showed that micronodular cirrhosis with hepatocarcinoma resembles human cirrhosis with hepatocarcinoma in both its biological and morphological aspects in close agreement with other studies[5-7]. Table 3 shows that TAA increases nuclear DNA content by 15% in rat liver, producing 50% cases with aneuploidy and 50% cases of hepatocarcinoma seen under microscopy was a suitable model for evaluating the effects of endotoxin on hepatocarcinogenesis.

DNA index representing a ratio of the DNA content in the treated rat to the mean DNA content of the diploid normal rats indicate precise malignity of hepatocarcinoma, together with aneuploidy rates in various groups, which were highly correlative to the incidence of hepatocarcinoma (the former r = 0.98, $P < 0.01$; the latter r = 0.96, $P < 0.01$). Moreover, the histologic studies with light and electron microscopes well confirmed the interesting findings. In the hepatocyte, clonal perpetuation of accumulated DNA aneuploid affecting growth regulatory genes eventually leads to a continuation of severe progressive abnormalities characterized by dysplasia, formation of adenoma and, finally, cancer[13]. In addition, plasma GGT activity, an enzyme of fetal hepatocytes, was a sign of poor differentiation and correlated with cancerous change (r = 0.93, P<0.05) and the degree of endotoxemia (r = 0.88, P<0.05).

Like Kupffer cells, splenic macrophages have a potential for endotoxin clearance by way of phagocytosis. In this study, rats undergoing splenectomy showed reduced endotoxin clearance and increased enterogenic endotoxin levels in systemic circulation by 37% (P<0.05). And rats with cirrhosis after LPS administration at a dose of 50μg/100g body weight daily for two months increased their endotoxemia by 24% (P<0.05), compared with the normal controls. When the rat endotoxemia increased,inhibition of hepatic DNA synthetic enzymes by endotoxin[12] resulted in a decrease of liver weights and formation of micronodules in comparison with the TAA control rats with macronodules. In addition, splenic macrophages have an ability to generate tumor necrosis factor-α and play an important role in immunosurveillance[13,14]. Therefore the down-regulation of Kupffer cell activity after splenectomy may have deleterious effects on host defenses[15].

This study shows that endotoxemia from orally administered LPS, during the last two months, and/or from splenectomy before commencement of the experiment, significantly enhanced hepatocarcinogenesis (P<0.02), based on the alterations of the DNA index. It seemed that the TAA model with splenectomy combining LPS administration was more deleterious to the development of hepatocarcinoma in rats than the TAA model treated with ST or LPS solely. In addition, it was noticed that the blood endotoxin levels in rats were significantly correlated with malignity (r = 0.96,P<0.01) and the incidence (r = 0.99, P<0.01) of hepatocarcinoma. If so, it may be of importance in the pathogenetic process of hepatocarcinoma and in clinical therapeutic strategy. However, patients with cirrhosis presenting relatively high endotoxemia[2] may have higher risk for development of hepatocarcinoma epidemiologically[3], but what are the possible mechanisms involved in the pathogenesis and whether this result may be extrapolated into humans await further studies.

REFERENCES

1 Han DW. A study of pathogenic mechanism of hepatic failure-hypothes is of intestinal endotoxemia.*Chin J Hepatol*,1995;3(3):46-49
2 Lumsden AB, Henderson JM, Kutner MH. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood. *Hepatology*,1988; 8(2):232-236
3 Niederau C, Fisher R, Sonnenberg A, Stremmel W, Tramprishch HJ, Strohmeyer G. Survival and causes of death in cirrhotic and noncirrhotic patients with primary hemochromatosis. *N Engl J Med*,1985;313(4):1256-1262
4 Han DW. Etiology of liver diseases. In: Han DW, ed. Hepatic pathophysiology. Vol 2.Taiyuan: Shanxi United Press of Universities, 1991
5 Mullen KD, McCullough MAJ. Problem with animal models of chronic liver disease: suggestions for improvement in standardization.*Hepatology*, 1989;9 (2):500-503
6 Noda S, Musumi S, Moriyama M, Yukiko K, Ohta M, Sugano T et al.Population of hepatic macrophages and response of perfused liver to platelet-activating fa-ctor during production of Thioacetamide induced cirrhosis in rats.*Hepatology*, 1996;24(2):412-418
7 Moreira E, Fontana L, Peviago JL, Medina FS, Gil A. Changes in fatty acid composition of plasma, liver microsomes, and erythrocytes in liver cirrhosis induced by oral intake of Thioacetamide in rats.*Hepatology*, 1995;21(1): 199-206
8 Porter WR. TAA-induced hepatic necrosis. *J Pharmacol Exp Ther*,1979;208 (1):386
9 Inada K, Endo S, Takahashi K, Suzuki M, Narita T, Yoshida T *et al*. Establishment of a new perchloric acid treatment method to allow determination of the total endotoxin content in human plasma by the limulus test and clinical application.*Microbiol Immunol*,1991;35(4):303-314
10 Xu KC, Meng XY, Wu JW. Diagnostic value of serum glutamyl transferase isoenzyme for hepatocellular carcinoma: a 10-year study. *Am J Gastroenterol*, 1992;87(8):91
11 Zuo LF, Guo JW, Liu JH. Flow cytometry DNA ploidy analysis in 208 cases of precancerous lesions.*Chin J Physic Med*,1992;14(1):31-34
12 Ikuyo T, Rieko N, Shosuke K. Effect of endotoxin on rat liver regeneration after partial hepatectomy.*Biochem International*,1992;27(4):1047-1050
13 Dragan YP and Pitot HC. The role of stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. *Carcinogenesis*, 1992;13 (5):379-750
14 Battafarano RJ, Burd RS, Kurrelmeyer KM, Rtaz CA, Dunn DL. Inhibition of splenic macrophage tumor necrosis factorα secretion in vivo by antilipopolysaccharide monoclonal antibodies.*Arch Surg*, 1994;129(2):179-186
15 Shohachi S, Satoaoshi N, Atsushi S, Takanori S, Hiroyuki K, Hiroyuki M *et al*. Role of Kupffer cells and the spleen in modulation of endotoxin-induced liver injury after partial hepatectomy.*Hepatology*, 1996;24(1):219-225

Stage Ⅱ surgical resection of hepatocellular carcinoma after TAE: a report of 38 cases *

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Subject headings carcinoma, hepatocellular/therapy; liver neoplasm/therapy; carcinoma, hepatocellular/surgery; liver neoplasms/surgery; embolization, therapeutic; hepatectomy

Abstract

AIM To evaluate the curative effect of stage II surgical resection of hepatocellular carcinoma after TAE.

METHODS Thirty-eight patients with unresectable hepatocellular carcinoma were treated by transcatheter arterial embolization (TAE). When the sizes of tumors were markedly reduced after TAE, stage II surgical resections were performed.

RESULTS Before TAE, the diameters of tumors were 12.84 cm ± 4.87 cm ($\bar{x}$ ± s), but reduced to 5.12 cm ± 1.82 cm ($\bar{x}$ ± s) after TAE (P<0.001). Pathologic examination of the resected specimens revealed obvious necrosis in most cases. After surgery, 26 patients were alive, with the longest survival of 96 months, twelve died and 10 had tumor recurrence.

CONCLUSION Patients in moderate and advanced stages of hepatocellular carcinoma after TAE should be treated surgically, but the indication must be controlled strictly.

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INTRODUCTION

Transcatheter arterial embolization (TAE) has been recognized as a new effective method for the treatment of moderate and advanced hepatocellular carcinoma[1-10]. Some patients are able to gain a chance of stage II surgical resection after TAE. But, what are the indications of stage II surgical resection? How about the curative effect? What are the influential factors? Up to date, there has been no report about these questions. A total of 1288 patients with advanced hepatocellular carcinoma were treated by TAE in our hospital from February 1987 to December 1992, 248 of them were followed up and 38 gained stage II surgical resection. The results are reported below in an attempt to explore the above mentioned questions.

MATERIALS AND METHODS

This group consisted of 38 patients with hepatocellular carcinoma diagnosed pathologically. They were treated by TAE via catheterization of hepatic artery on account of no indication for surgery. TAE procedures were carried out alike as previously reported[1,6,9,10]. The duration from initial TAE treatment to stage II surgical resection in this group was 4 weeks to 16 months, averaging 6.38 months. TAEs were done 1 to 9 times for each patient with an average of 3.6 times and at intervals of 50-60 days. According to hepatic arteriographic findings, 28 of 38 cases were classified as huge mass type or huge mass plus nodular type (the diameter of tumor ⩾10cm), 10 as nodular type (the diameter of tumor 5cm-9cm) and none as diffuse type.

RESULTS

Change of tumor size after TAE and pathologic examination

Before TAE, the diameters of tumors were 12.84 cm±4.87cm ($\bar{x}$ ± s) and after TAE decreased to 5.12cm ± 1.82cm ($\bar{x}$ ± s), (P<0.001) (Figures 1-6). Tumors were removed in 36 cases, but not in the remaining 2 because of spreading to diaphragm and lesser epiploon, absolute ethanol was injected directly into the tumors with ligation of feeding hepatic artery. Liver tumor capsules were intact in 19 of 36 resected specimens observed with naked eyes, the capsules were not intact in 14, and none in 3. Under light microscope, there was complete necrosis of tumor cells in 12 cases, obvious necrosis

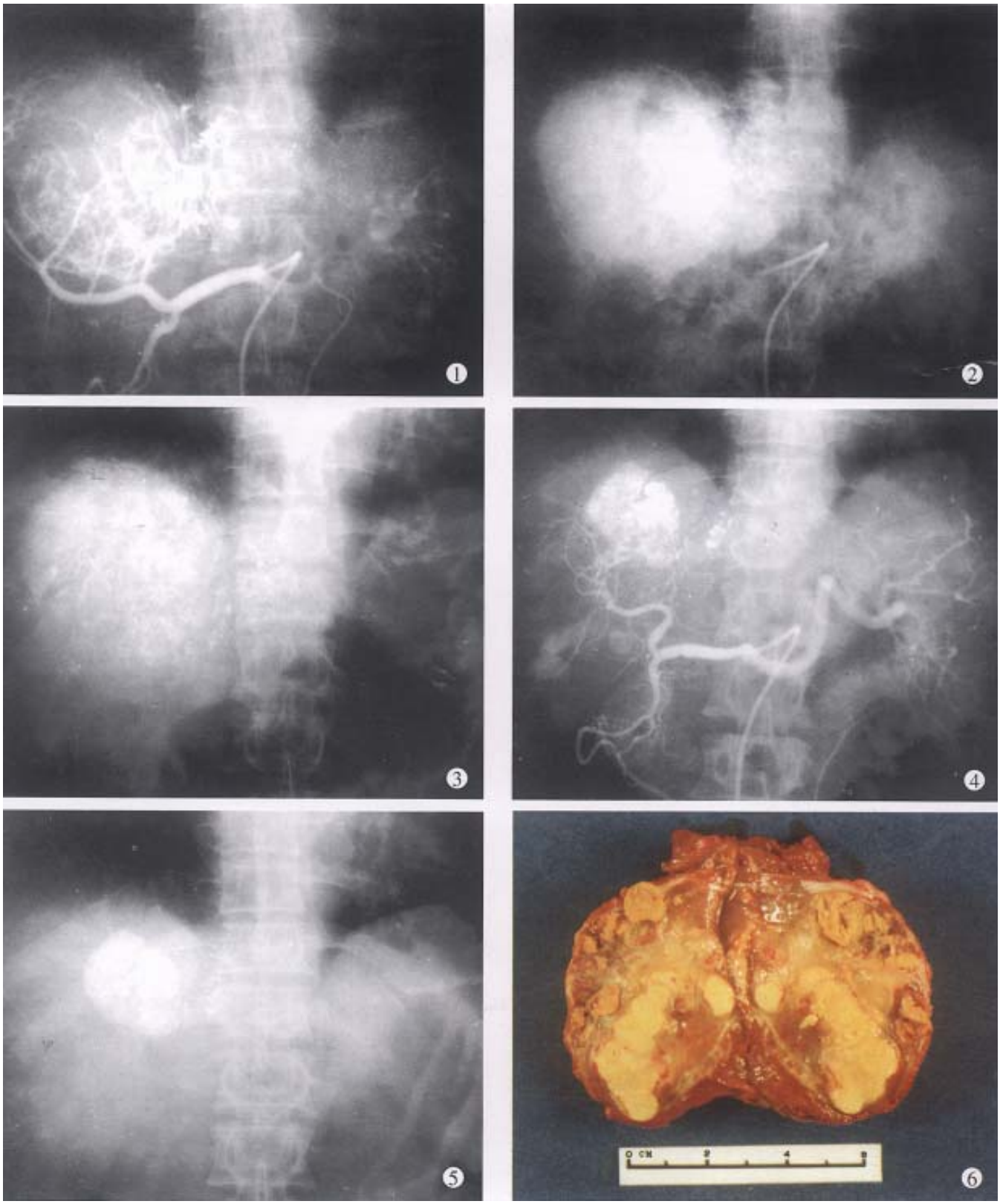

Figure 1 The hepatic arteriography shows a large tumor with hypervasculars in the right lobe of liver.
Figure 2 Abdominal plain film immediately after TAE shows accumulation of lipiodol within the tumor.
Figure 3 A parenchymal phase of the hepatic arteriography. Tumor mass staining is well visualized.
Figure 4 The hepatic angiography after three times of TAE shows occluded branches of right hepatic artery with some collateral vessels and remarkable tumor reduction.
Figure 5 Abdominal plain film after four times of TAE shows deposition of lipiodol within the tumor. The diameter of tumor was reduced to 4cm.
Figure 6 After four times of TAE, the tumor was resected. The specimen showed necrosis of tumor and fibrotic capsule.

in most parts of the tumor with a few living tumor cells in 20 cases, and partial necrosis with parts of tumor cells actively growing in 4 cases. There were inflammatory reaction and different degrees of fibrotic proliferation around the necrotic tissues. During operation, mild liver cirrhosis was seen in 11 cases while moderate and serious ones in 21 and 4 cases respectively, and none in 2.

The survival of patients after stage II surgical resection

So far, 26 of 38 patients have been alive, 22 of 26 being healthy. The average survival was 24.6 months, and the longest survival period has reached 96 months. Tumor recurred in 4 cases, 2 had bone metastasis one month after surgery, one was found to have multiple intrahepatic tumor nodules and one had elevated AFP values with right liver recurrent tumor of 4.5cm×5.2cm in diameter, occurring 3 months and 19 months after surgery, respectively. Twelve patients died, 2 died separately from coronary arterial embolization and massive upper digestive tract bleeding one week after surgery, 8 died from recurrence of liver tumor, and the other 2 died of serious liver cirrhosis and liver function failure 4 and 16 months after surgery. The time between surgery and death was 4-28 months, with an average of 11.5 months (excluding 2 deaths one week after surgery).

Recurrence condition of liver tumors after surgery

Tumor recurred after surgery in 10 patients, 8 of the 10 recurrences were within liver, whereas 2 had bone metastasis. So far, 8 patients have died. Postoperative tumor recurrence time was 1 to 21 months with an average of 7.2 months. Cancerous emboli were found within the main trunks of portal vein or its large branches in the 10 patients, and 1 of them had also cancerous emboli in the right hepatic vein.

DISCUSSION

TAE effectiveness depends on the thoroughness of embolization

In our 38 patients, the sizes of liver tumors were markedly reduced after TAE. Pathologic examination of the resected specimens revealed obvious necrosis in most parts of tumor masses in nearly all the cases and complete necrosis in a few ones[6,9,10]. All these indicated the good therapeutic effects of TAE for hepatocellular tumor. The reason for the better results obtained in this group by TAE was the thorough embolization of tumor vessels and feeding arteries, as shown by the accumulation of large amount of lipiodol in all or most parts of tumor mass on CT scanning. Besides, most of the tumors in this group were of huge mass type or single nodule type. Furthermore, the lipiodol emulsion containing anticancer drugs obstructed the blood supplying to the tumors, resulting in ischemic necrosis[2,5,8] on one hand, and on the other hand, the lipiodol emulsion continued to release anticancer drugs for killing tumor cells which were already in an ischemic and more vulnerable state[6,9,10]. Therefore, the thoroughness of embolization for liver tumors directly influenced the effect. As far as the patients' liver function would be tolerated, the embolization procedure should be undertaken to fully fill the tumor mass with lipiodol emulsion during each TAE, especially for the first time[9,10].

Necessity of stage II surgical resection

The surgical specimens from the 38 cases revealed that most parts of the liver tumors were necrotic, but a few living cancerous cells still remained, which might cause tumor recurrence or metastasis later[6,9,10]. On the other hand, after several times of TAE procedure, the main tumor feeding arteries had been obstructed and more collateral vessels thus formed, causing difficulties in super-selective catheterization via the collateral vessels and continuous TAE. The majority of patients were only treated by arterial infusion chemotherapy, which was usually ineffective in controlling tumor growth. Therefore, the liver tumor which had been markedly reduced in size after TAE should be removed further by stage II surgery. Thus, the prognosis would be much improved.

Prevention and reduction of tumor recurrence after surgery

In this group, ten patients had liver tumor recurrence on an average of 7.2 months after stage II surgery (10/38, or 26.3%). There were three types of recurrences: ① 10 patients showed all cancerous emboli in the main trunks of portal veins and their bigger branches, with 1 case also having cancerous emboli in the right hepatic vein; ② in some cases, severaltumor nodules were still present besides the main tumor mass; and ③ in a few cases, merely palliative surgical resections were performed because the tumor sizes found during operation were still too large. Therefore, the indication for stage II surgical resection after TAE should be controlled strictly. The contraindications are as follows: hepatic arteriogram and CT scanning reveal main tumor mass with several satellite tumor nodes which can not be removed completely by surgery; the diameter of tumor is over 5 cm, which is only resected palliatively; cancerous emboli within the main trunk of portal vein or its large branches or large hepatic venous branches; distant metastasis; and serious liver cirrhosis.

REFERENCES

1 Yamada R, Sato M, Kawabata M, Nakatsuka H, Nakamura K, Takashima S. Hepatic artery embolization in 120 patients with unresectable hepatoma. *Radiology*,1983;148(2):397-401

2 Nakakuma K, Tashiro S, Hiraoka T, Uemura K, Konno T, Miyauchi Y. Studies on anticancer treatment with an oily anticancer drug injected into the ligated feeding hepatic artery for liver cancer.*Cancer*, 1983;52(12):2193-2200

3 Ohishi H, Uchida H, Yoshimura H, Ohue J, Katsuragi M, Matsuo N *et al*. Hepatocellular carcinoma detected by iodized oil. Use of anticancer agents. *Radiology*,1985;154(1):25-29

4 Nakakuma K, Tashiro S, Hiraoka T, Ogata K, Ootsuka K. Hepatocellular carcinoma and metastatic cancer detected by iodized oil. *Radiology*, 1985;154(1): 15-17

5 Miller DL, Timothy J, O'Leary, Girton M, RT. Distribution of iodized oil within the liver after hepatic arterial injection. *Radiology*, 1987;162(3):849-852

6 Kenematsu T, Furuta T, Takenaka K, Matsumata T, Yoshida Y, Nishizaki T *et al*. A 5 year experience of lipiodolization: selective regional chemotherapy for 200 patients with hepatocellular carcinoma. *Hepatology*, 1989;10(1):98-102

7 Uchida H, Ohishi H, Matsuo N, Nishimine K, Ohue S, Nishimura Y *et al*. Transcatheter hepatic segmental arterial embolization using lipiodol mixed with an anticancer drug and gelfoam particles for hepatocellular carcinoma.*Cardiovasc Intervent Radiol*,1990;13(3):140-145

8 Yamada R, Kishi K, Sonomura T. Transcatheter arterial embolization in unresectable hepatocellular carcinoma.*Cardiovasc Intervent Radiol*, 1990;13(2): 135-137

9 Lin G, Wang JH, Gu ZM, Yan ZP, Wang XL. Hepatic arterial infusion chemotherapy and embolization in the treatment of moderate and advanced stages of hepatic carcinoma: therapeutic effects and some influential factors. *Chin J Radiol*,1992;26(5):311-315

10 Wang JH, Lin G, Yan ZP, Wang XL. Hepatic arterial infusion chemotherapy plus embolization for unresectable liver cancer: a report of 40 patients. *Chin J Oncol*, 1992;14(4):276-278

Regulatory effects of lipopolysaccharide in murine macrophage proliferation

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Subject headings lipopolysaccharide; macrophage; granulocyte-macrophage colony-stimulating factor; transforming growth factor beta; interferon-γ; polymerase chain reaction; cytokines; RNA, messenger

Abstract

AIMS To study the regulatory effects of bacterial lipopolysaccharide (LPS) in murine macrophage proliferation.

METHODS Using murine peritoneal exudate macrophage (PEM) and macrophage cell line J_{774}A.1 as targets, LPS effects on M-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated macrophage colony-forming cells (CFU-M) were detected. 125I-GM-CSF receptor binding assay was used to examine LPS regulation on GM-CSF receptor expression. RT-PCR was employed to test TGF-β_1 inhibition on IFN-γ mRNA expression on macrophage induced by LPS.

RESULTS Without direct effect on macrophage proliferation, LPS could inhibit the macrophage proliferation stimulated by GM-CSF. However, with the concomitant existence of GM-CSF and TGF-β_1, the LPS inhibitory effect was eliminated. RT-PCR analysis indicated that the strongest macrophage growth inhibitory factor IFN-γ mRNA expression in macrophage induced by LPS was remarkably suppressed by TGF-β_1, 125I-GM-CSF receptor binding assay showed that LPS could enhance GM-CSF receptor expression likewise as TGF-β_1.

CONCLUSIONS LPS is involved in the network of macrophage proliferative regulation by multiple cytokines, displaying inhibitory and stimulatory effects based on the coexisting cytokines.

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INTRODUCTION

Lipopolysaccharide (LPS), main component of Gram[a2]negative bacterial endotoxin, is involved in immune reaction by intriguing macrophage and B lymphocyte. It has been demonstrated that interferon-γ (IFN-γ) is the strongest inhibitor of macrophage colony formation[1,2,6]. Therefore, LPS is thought to inhibit macrophage growth. Recent reports indicated that LPS at low concentration (< 0.1μg/L) had stimulatory effects in some hematopoietic cells[3,4]. So, the effects of LPS in cell growth regulation is multifunctional. Tissue macrophges migrated from peripheral monocytes are the main targets of LPS and participate in immune response by LPS activation. Besides, macrophages are known to be able to form colony under M-GSF and GM-CSF stimulations. In this study, LPS effects in macrophage proliferation was investigated.

MATERIALS AND METHODS

Animal and cell

Murine peritoneal exudate macrophages (PEM) in 6-8-week old CsH/HeJ mice were induced by thioglocallate medium J_{774}A.1 was obtained from ATCC.

Reagent

rMuG-M-CSF was obtained from Immunex Co, rHum-M-CSF as gift from Cetus Co, rHuTGF-β_1 from NCI, and LPS was purchased from List Co, and RT-PCR Kit and primers from Cetus Co.

GM-CSF and M-CSF iondination

Bolton-Hunter method was used for GM-CSF iondination and chlormate T method for M-CSF iondination. Receptor binding assay was undertaken according to Reference 5.

Macrophage colony formation (CFU-M)

PEM 1000/ml in a medium containing 10% FCS (Hyclone Co.) was cultured in 24-well plates at 5% CO2 atomasphere and 37℃ temperature. rMuGM-CSF (0.5 μg/L), LPS (0.01 μg/L - 100 μg/L) or rHuTGF-β_1 (0.5 μg/L) were added at different intervals. After 7-9 days, CFU-M (cell number > 50) was formed and calculated by a Coulter counter.

RT-PCR

Reverse-transcription polymerase chain reaction (RT-PCR) was applied to examine cytokine mRNA expression. Experimental detail was referred to

Reference 5. The IFN-γ and β-actin primers are as follows:

IFN-γ 5'-primer(71):TGAACGCTACACACTGCTTCTTGG
3'primer(530):CGACTCCCTTTTCCGCTTCCTAG
β-actin 5'-primer(25):GTGGGCCGCTCTAGGCACCAA
3'-primer(564):CTCTTTGATGTCACGCACGATTTC

RESULTS

LPS effects in murine macrophage growth

PEM was potential to form CFU-M in vitro under optimal GM-CSF stimulation. Figure 1 shows that LPS at a concentration of lower than 1.0μg/L considerably inhibited macrophage growth and this inhibition was typically dose-dependent. However, with concomitant addition of rHuTGF-β_1 (0.5μg/L), LPS inhibition was reversed to be stimulatory while LPS and TGF-β_1 had no direct effects in CFU-M (Figure 2). Our previous paper reported that TGF-β_1 enhancement on GM-CSF stimulation was associated with the increase of GM-CSF receptor number[6]. Accordingly, LPS effect in GM-CSF receptor regulation was further examined.

LPS effects in GM^{a2}CSF receptor number

LPS could remarkably decrease M-CSF receptor number within a short time (37℃, 1h), but without effect on GM-CSF. After LPS treatment in PEM was prolonged to 24 hours, GM-CSF receptor number was increased by over 80%, being time-dose dependent (Figure 3).

LPS effects in murine macrophage cell line

Although murine macrophage cell line J_{774}A.1 autonomous growth was inhibited by LPS, its membrane GM-CSF receptor number was enhanced (Figure 4). These results confirmed the upregulation of LPS in GM-CSF receptor expression.

The inhibition of IFN-γ mRNA expression by TGF-β_1

IFN-γ mRNA expression in PEM was examined with (RT-PCR) (Figure 5). Consequently, IFN-γ mRNA was expressed after LPS induction in PEM for 2 hours, which was totally suppressed by TGF-β_1 treatment for 2 hours.

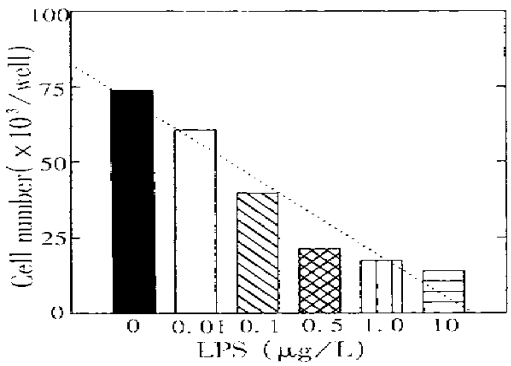

Figure 1 Dose-dependence of LPS inhibitory effects in macrophage growth.

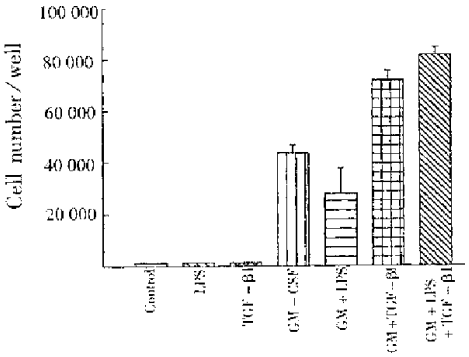

Figure 2 Effects of LPS, TGF-β_1 and GM-CSF in macrophage growth in co-stimulation.

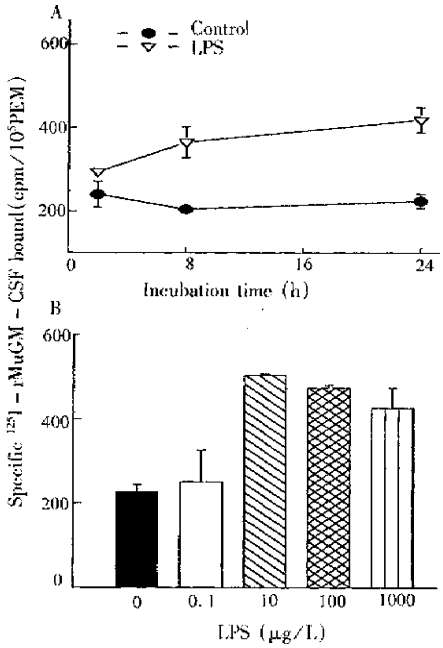

Figure 3 Enhancement of GM-CSF receptor number by LPS. A: Time-dose of LPS at 5.0μg/L concentration; B: Effects of different doses of LPS on GM-CSF receptor number (37℃, 24h).

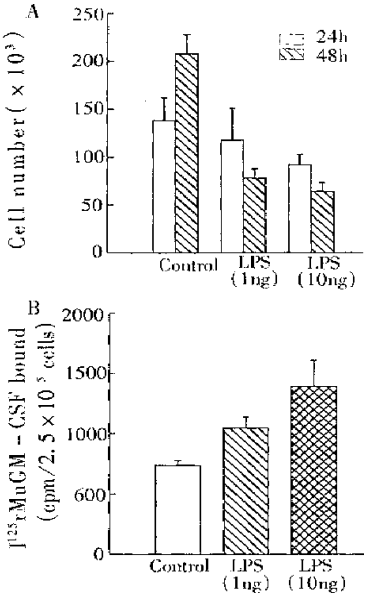

Figure 4 A: LPS inhibition in J_{774}.1 autonomous growth. B: GM-CSF receptor number increased by LPS in J_{774}.1.

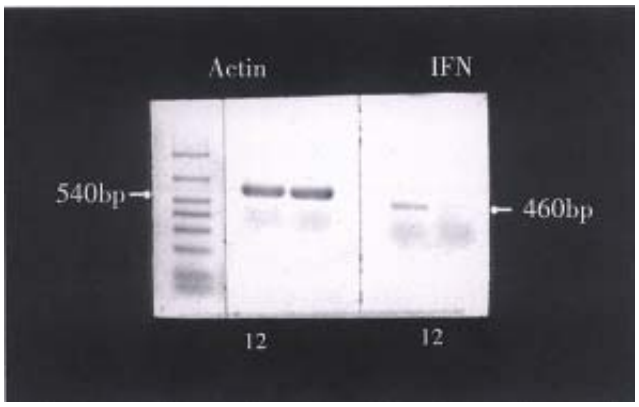

Figure 5 TGF-β_1 inhibition on IFN-¦Ã mRNA expression. Lane 1: LPS (1.0μg/L) induction for 2 hours. Lane 2: TGF-β1 (1.0 μg/L) treatment for 2 hours.

DISCUSSION

Macrophages play an important role in a series of physiological and pathogenic processes, such as anti-infection, anti-tumor, immune response and inflammation. Macrophage growth regulation is modulated by multiple cytokines. Among them only GM-CSF and M-CSF can stimulate macrophage colony formation. Others (IL-4, TNF-α, L-3, IFN-γ, TGF-β_1) influence macrophage growth by regulating M-CSF or GM-CSF receptor number and expression[4-7]. Bacterial lipopolysaccharide mainly activates macrophage and plays a certain role in its proliferation. LPS at in vivo concentration of 0.1μg/L-10μg/L, can inhibit GM-CSF stimulated macrophage growth but without direct effect. Interestingly, the addition of TGF-β_1 turns the LPS effect from inhibitory to stimulatory.Receptor binding assay indicates that LPS and TGF-β_1 can both enhance GM-CSF receptor number, probably because of effects of the three modulators (GM-CSF, LPS and TGF-β_1) in macrophage growth. As IFN-γ is induced by LPS and is the strongest autocrine inhibitor of macrophage growth. TGF-β_1 may suppress IFN-γ expression in macrophage by RT-PCR technique. According to our results, a regulating network of LPS in macrophage proliferation is presumed. LPS is capable of increasing GM-CSF receptor number, but IFN-γ displays stronger inhibition on macrophage growth. With the coexitence of TGF-β_1, IFN-γ expression is remarkably suppressed and GM-CSF receptor number is further enhanced. As a result, the concomitant presence of LPS, TGF-β_1 and GM-CSF stimulates macrophage proliferation. Our findings could lead to a conclusion that it is necessary to further reveal the complexity, complementarity and continuity of three or more cytokines in macrophage proliferation.

REFERENCES

1 Rennick D, Yang G, Gemmell L, Lee F. Control of hemapoiesis by a bone marrow stromal cell clone: lipopolysaccharide and interleukin-1 inducible products of colony stimulating factor.*Blood*,1987;69(2):682-691
2 Ding AH, Nathan CF. Trace level of bacterial lipopolysaccharide prevents interferon-α or tumor necrosis factor alpha from enhancing mouse peritoneal macrophage respiration bursh capacity.*J Immunol*, 1987;139(6):1971-1977
3 Moore RN, Steel PS, Mannel DN, Mergenhagen SE. Role of lipopolysaccharide in regulating colony stimulating factor-dependent macrophage proliferation in vitro.*Infect Immunity*,1980;30(3):797-804
4 Yui S, Yamazaki M. Induction of macrophage growth by Lipids. *J Immunol*, 1986;136(2):334-339
5 Fan K, Ruan Q, Sensenbrenner L, Chen B. Upregulation of granulocyte-macrophage colony stimulating factor (GM-CSF) receptors in murine peritoneal exudate macrophages by both GM-CSF and IL-3. *J Immunol*, 1992;149(1): 96-102
6 Chen BD, Chou TH, Ratanatharathorn V. Expresion of gamma interferon receptor in murine bone-marrow derived macrophages associated with macrophage differentiation: evidence of gamma-interferon receptors in the regulation of macrophage proliferation.*J Cell Physiol*, 1987;133(2):313
7 Takway JP, DeFranco AL. Pertussin toxin inhibition of B cell and macrophage response to bacterial lipopolysaccharide. *Science*,1986;234(6):743

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Evaluation of various solutions for small bowel graft preservation *

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Subject headings intestine, small/transplantation; organ preservation; adenosine triphosphate; intestine, small/transplatation; Na (+)-K(+)-exchanging ATPase; adenine nucleotides

Abstract

AIM To elucidate the effect of various solutions for small bowel graft preservation in pigs under hypothermic storage.

METHODS The swine segmental small bowel graft was autotransplanted after it was preserved with lactated Ringeriäs (LR), Euro-Collins (EC), hyperosmolarity citrate adenine (HC-A) and WMO-1 solutions for 10, 18 and 24 hours, respectively. The recipient survival rate, morphological structure, graft mucosal energy substances and Na + K+ ATPase activity were studied, and graft absorption was estimated with D-xylose absorption test.

RESULTS The morphological study of the grafts preserved with LR or HC-A solution for 10 hours or with EC and WMO-1 solution for 18 hours was normal 6 days after operation. Mucosal ATP, total adenine nucleotides (TAN) contents and Na^+-K^+ATPase activity of the graft preserved with EC or WMO solution were higher than that of the graft preserved with LR or HC-A solution. Serum level of D-xylose was higher in EC and WMO-1 groups than in LR and HC-A groups when the graft was preserved for 24 hours.

CONCLUSIONS EC and WMO-1 solutions can preserve the swine small bowel up to 18 hours, which are superior to LR and HC-A solutions.

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INTRODUCTION

One of the problems accompanying successful clinical small bowel transplantation is the graft preservation, for the intestinal mucosa is probably the most sensitive tissue to ischemia in the body. The effective time of cold storage for small bowel graft (< 8 hours) is much shorter than that of other organs, and optimal preservation solution is not yet clearly defined for small bowel grafts. In this study, the swine small bowel was preserved with lactated Ringer's (LR), hyperosmolarity citrate adenine (HC-A), Euro-Collins (EC) and WMO-1 solutions to elucidate which is appropriate for swine small bowel preservation.

MATERIALS AND METHODS

Study design

Outbred pigs weighing 18.5kg-22.5kg were used. The pigs were fasted for 24 hours before operation, and general anesthesia was achieved with intravenous sodium pentobarbital.

Heterotopic segmental small bowel autotransplantation was performed as described previously[1]. The small bowel was sectioned before the ileocecal valve and mid-small intestine. The superior mesenteric artery of the graft was perfused with 400ml-500ml LR, EC, HC-A or WMO-1 solutions, and stored in the same solution at 4℃ for 10, 18 and 24 hours, respectively. The perfusion pressure was 10.8kPa-11.8kPa, and perfusion time was 20 minutes. The lumen was perfused with metronidzzole solution at 4¡æ after perservation, and the small bowel graft was autotransplanted. Both ends of the grafts were brought out as stomas (Thiry-Vella loops). Intestinal continuity was restored with two end-to-end anastomoses. The experimental groups and preservation time are shown in Table 1.

Recipient survival rate

The recipient survival rateon the 21st postoperative day (POD) was observed.

Table 1 Experimental groups and preservation time

Groups	Preservation time		
	10 h	18 h	24 h
LR	5	5	6
EC	5	7	6
HC-A	6	5	5
WMO-1	5	6	5

Mucosal morphology

Biopsy specimens obtained from the graft stomas, fixed in 10% neutral formation and embedded in paraffin, and sections were cut at 4 μm thickness, and stained with haemotoxylin and eosin. All specimens were observed under light microscopy.

Mucosal energy substances

Small bowel graft energy phosphates (ATD, ADP and AMP) were determined by high performance liquid chromatography (HPLC) as described previously[2]. The mucosa (weighing about 0.5g) was separated from the seromuscular wall and stored in liquid nitrogen. Atkinson energy charge (AEC) was calculated, AEC=(ATP+0.5ADP)/TAN, total adenine nucleotides (TAN) ATP+ ADP+ AMP.

Mucosal Na^+-K^+ ATPase activity

Small intestinal mucosal Na^+-K^+ ATPase activity was determined using Zemelman's method[3]. The mucosa was separated and stored by the above-mentioned method.

Absorptive function

Absorptive function of the graft was estimated with D-xylose absorption test. Five g D-xylose (dissolved in 100ml normal salt solution) was irrigated into the graft from the proximal stoma, and serum D-xylose level was determined by Eberts' method[4].

Statistical analysis

All results were expressed as $\bar{x} \pm s$, statistical variances were made by Student's *t* test or χ^2 analysis, differences were considered significant if $P<0.05$.

RESULTS

Recipient survival rate

Six animals died because of failure in vascular anastomosis. Five animals were observed in each group. The survival rate on the 21st POD was similar in the graft preserved with each of the four solutions either for 10 or 18 hours. When the graft was preserved for 24 hours, the 21st POD survival rate was lower in LR group than in EC and WMO-1 groups ($P<0.05$, Table 2).

Morphological study

Epithelial necrosis and edema occurred by all the solutions. On the 3rd or the 4th POD, the grafts became normal in all groups histologically when the graft was preserved with each of four solutions for 10 hours 6 or 7 days after operation. When the graft was preserved for 18 or 24 hours, more severe tissue reperfusion injury was seen after revasculation in LR and HC-A groups than in EC and WMO-1 groups. On the 21st POD mucosa histological structure of the grafts preserved with EC and WMO-1 solutions for 18 hours were normal. Necrosis was present in the graft preserved with LR and HC-A solutions. When the graft was preserved with each of the four solutions for 24 hours, the mucosal histological structure can not recover to normal.

Mucosal energy substances

The concentration of phosphorylated adenine nucleotides was similar in the graft preserved with the four solutions for 10 hours. Cold storage significantly reduced the contents of ATP and TAN (Figures 1 and 2). Tissue contents of ATP and TAN were significantly higher in the graft perserved with EC and WHO-1 solutions after 24 hours of cold storage. The energy change as a parameter for the freely available energy in the cell was significantly higher in EC and WMO-1 groups (0.66nmol/g ± 0.03nmol/g wet weight, 0.63 nmol/g ± 0.03 nmol/g wet weight) than in LR and HC-A groups (0.55 nmol/g ± 0.04 nmol/g wet weight, 0.58 mol/g ± 0.03 nmol/g wet weight).

Mucosal Na^+-K^+ ATPase activity

Mucosal Na^+-K^+ ATPase activity remained unchanged during cold storage despite storage time and preservation solution. Na^+-K^+ ATPase activity was decreased after 30 minutes of reperfusion (Table 3) in all the grafts. Mucosal Na^+-K^+ ATPase activity was significantly lower in LR and HC groups than in EC and WMO-1 groups after 24 hours of cold storage.

Table 2 Recipient survival rate on the 21st POD (%)

Groups	Preservation time		
	10 h	18 h	24 h
LR	100	60	0[a]
EC	100	100	60
HC-A	100	80	20
WMO-1	100	100	60

[a]$P<0.05$, compared with EC and WMO-1 groups

Table 3 Mucosal Na^+-K^+ ATPase activity after 30 min of reperfusion (μmol Pi/mg protein·h, $\bar{x}\pm s$)

	Control	10 h	18 h	24 h
LR	15.15±3.71	8.29±2.89	5.79±1.57	1.15±0.47
EC	16.16±2.59	9.23±3.47	6.89±2.62	4.27±1.34[a]
HC-A	14.74±1.56	9.79±2.15	5.79±1.69	2.92±1.39
WMO-1	13.49±3.92	9.34±1.29	6.03±1.27	5.06±1.27[a]

[a]$P<0.05$, compared with LR and HC-A groups

Absorptive function

After 10 and 18 hours of cold storage, serum level of D-xylose was similar in all groups while after 24 hours it was higher in EC and WMO-1 groups than in LR and HC-A groups (Figure 3).

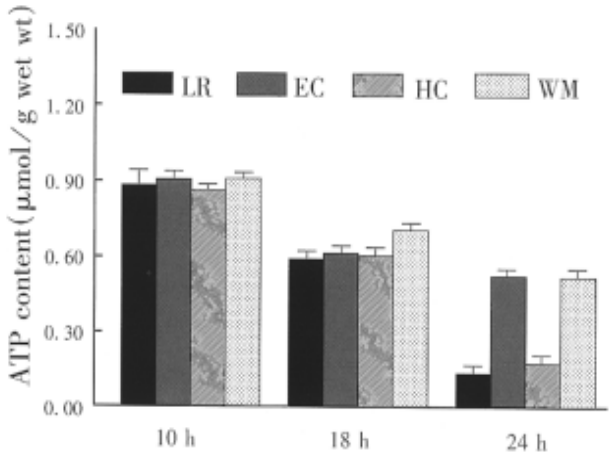

Figure 1 Mucosal concentration of ATP.

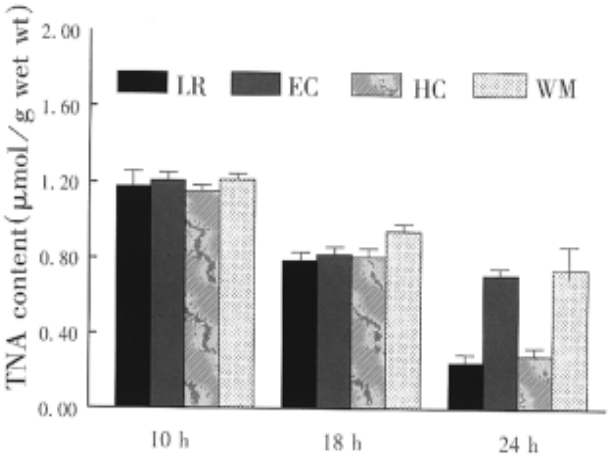

Figure 2 Mucosal concentration of TNA.

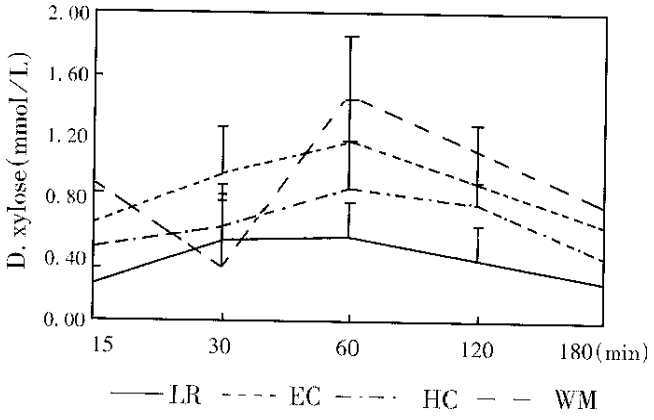

Figure 3 Results of D-xylose absorption test after 24 hours of cold storage.

DISCUSSION

Simple cold storage is the most practical method in organ preservation. Several solutions have been used in organ preservation, however, the optimal one is not clearly defined for small bowel grafts. A recently developed organ preservation solution, UW (University of Wisconsin) has provided 72 hours preservation for pancreas and kidney, and 30 hours or longer for the liver, but provided only 8-10 hours preservation for small bowel in human[5].

LR solution, an extracellular fluid, is widely used in experimental small bowel preservation. Luther *et al*[6] and Zhang *et al*[7] reported that LR solution is superior to UW and EC solutions for small bowel storage in the rat model. In our studies, no animal receiving the graft preserved for 24 hours survived more than 21 days. The recipient survival rate in EC and WMO-1 groups was 60%, respectively. Mucosal contents of ATP and TAN, and Na^+-K^+ ATPase activity in EC and WMO-1 groups were higher than in LR and HC-A groups after 24 hours of cold storage. Our results indicated that EC and WMO-1 solutions are superior to LR solution for small bowel preservation in the pig model.

HC-A solution, a modified Ross solution, contains adenine, precursor for ATP production, and its osmolarity was reduced from 400 to 380mOsm/L. The solution has provided 48 hours or longer preservation for the cadaveric kidneys. In our study, mucosal contents of ATP and TAN and Na^+-K^+ ATP ase activity in HC-A preserved small bowel was not higher than in EC and WMO-1 preserved one, so adenine added into the HC-A solution could neither improve the ATP production nor inhibit the tissue ATP degradation.

WMO-1 solution, containing ATP and Ca^{2+} could provide 48 hours preservation for the liver in the rat model. It is believed that ATP in WMO-1 solution could increase the energy state of tissue and prevent ATP degradation and the calcium could stabilize the membranes. Our study indicated that mucosal contents of ATP and TAN were not higher in WMO-1 preserved small bowel than in EC- preserved one. Mucosal Na^+-K^+ ATPase activity was similar in the two groups, there fore, ATP added into WMO-1 solution could not prevent ATP degradation in tissues. It was not introduced in latest-developed organ preservation solution, such as UW. In the healthy cells, cytosolic calcium is at very low level (10^{-7}mmol/L), and hypothermic preservation may disrupt the cell membrane, resulting in influx of calcium into the cell. Elevated intracellular calcium was also shown to potentiate cell damage[8], therefore the effects of ATP and Ca^{2+} in the WMO-1 solution should be studied.

As shown previously, the EC solution is a standard organ preservation solution and has been widely used in liver, kidney, pancreas and small bowel

transplantations[5]. In this study, EC solution is superior to HC-A and LR solutions, and mucosal contents of ATP and TAN and Na^+-K^+ ATPase activity were higher in EC group than in LR and HC-A groups and were similar to that of WMO-1 group.

Recently, a direct correlation between ATP content during cold preservation and outcome in animal and clinical organ transplantation has been reported[9]. Na^+-K^+ ATPase is an important membrane-bound enzyme that acts by active extrusion of Na^+ from the intracellular compartment to counterbalance the osmotic effect of impermeable intracellular macromolecules. We investigated the effect of four solutions in recipient survival rate, mucosal energy substances, Na^+-K^+ ATPase activity and absorption of the graft. The results showed that Na^+-K^+ ATPase activity maintained unchanged during cold storage and reduced significantly after reperfusion but the contents of ATP and TAN decreased during cold storage. There was a direct correlationship between contents of ATP and TAN after cold storage and Na^+-K^+ ATPase activity after reperfusion and recipient outcome after transplantation. EC and WMO-1 solutions were superior to LR and HC-A solutions for small bowel graft.

In conclusion, the swine small bowel can be successfully preserved in good condition for 18 hours using simple cold storage in EC and WMO-1 solutions as assessed by the recipient survival rate, mucosal energy substances, Na^+-K^+ ATPase activity, and D-xylose absorption test and the EC and WMO-1 solutions are superior to LR and HC-A solutions for preservation of small bowel in the pig model.

REFERENCES

1 Li N, Li JS, Liao CX, Li YS, Wu XH. Segmental small bowel allotransplantation in pigs.*Chin J Organ Transplant*,1993;14(1):2-4

2 Schweinsberg PD, Loo TL. Simultaneous analysis of ATP, ADP, AMP and other purines in human erythrocyte by high-performance liquid chromatography. *J Chrom*,1980;18(1):103-107

3 Zemelman BV, Alker A, Chu SW. Expession and development regulation of Na^+-K^+ adenine trisphosphates in the small intestine. *J Clin Invest*, 1992;90(3): 1016-1022

4 Eberts JL, Sample KHB. Cilick MR, Ellis GM. A simplified colometric micromethod for xylose in serum or urine with pholoroglucind. *Clin Chem*, 1979;25 (8):1440-1443

5 Tolodo-pereyra LH. Small bowel preservation, evolution and ideas, and current concepts. *Transplant Proc*,1992;24(3):1083-1084

6 Luther B, Lehman C, David H, Klinnert J. Preservation of isolated intestinal segment using the University of Wisconsin solution. *Transplant Proc*,1991;23 (5):2459

7 Zhang S, Koluda Y, Neato Em. Biochemical evidence on mucosal damage of intestinal graft during cold preservation in University of Wisconsin, Euro-collins and lactated Ringer's solution.*Transplant Proc*, 1994;24(6):1087-1088

8 Clavien PA, Harvey PRC, Strasberg SM. Preservation and reperfusion injury in liver allografts.*Transplantation*, 1992;53(5):957-978

9 Harvey PRC, In S, Mckeown CMB, Petranka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia.*Transplantation*,1988;45(6):1016-1020

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Experimental research on production and uptake sites of TNFα in rats with acute hemorrhagic necrotic pancreatitis *

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Subject headings tumor necrosis factor/blood; endotoxius/blood; pancreatitis/blood; acute diseases; disease models, animal; enzyme-linked immunosorbent assay; pancreas/pathology; necrosis

Abstract

AIM To determine the site of production and uptake of tumor necrotic factor alpha (TNFα), and evaluate the relationship between serum TNFα and plasma endotoxin (ET) in rats with acute hemorrhagic necrotic pancreatitis (AHNP).

METHODS Sprague Dawley rats were divided into AHNP group and control group (n = 12). AHNP model was induced by retrograde injection of 5% sodium taurocholate via pancreatic bile duct. The blood samples were obtained through portal vein 2 and 6 hours after the operation.

RESULTS The contents of TNFα in portal vein were increased rapidly in the development of AHNP. They were lower in hepatic vein (280.59±20.02) and femoral artery (310.82±7.97) than in portal vein (354.91±25.50) (P<0.05), and higher in femoral artery than in hepatic vein 6 hours after the operation (P<0.05). TNFα level in plasma was increased significantly when ET level in portal vein showed no increase.

CONCLUSION Pancreas, spleen, liver, intestinal tract and lung are the main organs to produce TNFα, and liver is also an important site for TNFα uptake in the development of AHNP. Plasma endotoxin is not a trigger for TNFα release in rats with AHNP.

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INTRODUCTION

Recent studies have indicated that during the pathogenesis of acute hemorrhagic necrotic pancreatitis (AHNP), tumor necrotic factor alpha (TNFα) and endotoxemia play important roles in the progression of pancreatic inflammation and the damage of ex-pancreatic vital organs£Û1£Ý. The purpose of this paper is to determine the production and uptake sites of TNFα, and the relationship between TNFα and plasma endotoxin (ET) in rats with AHNP, thus providing a direct theoretical basis for the treatment of AHNP by blocking the production of TNFα and clearing the secreted TNFα in plasma.

MATERIALS AND METHODS

Animals and grouping

Twenty-four Sprague-Dawley (SD) rats weighing 295g-320g were divided randomly into AHNP group and control group (12 animals for each group).

Experimental design

SD rats were fasted for 24 hours, and were anesthetized by intraperitoneal injection of sodium pantobarbital (30mg/kg). Normal saline (0.9%) was injected into each rat through a PE-50 placed catheter in femoral vein. Sodium taurocholate (5% 1.5ml/kg) were infused retrogradely to AHNP rats through pancreatic bile duct under a microsurgical procedure, after 5min - 10min, pancreatic edema and dotted bleeding occurred. Isovolume of 0.9% normal solution was infused to the pancreatic bile duct of control rats using the same method, and no abnormality was noticed 2 and 6 hours after the operation. Blood samples from hepatic vein, portal vein and femoral artery were harvested, at these time points, hemorrhage, necrosis of pancreas and hydremic ascites (5ml-10ml) were found in AHNP- rats, while no abnomality was seen in the control rats.

Determination of serum TNFα.

TNFα in portal vein, hepatic vein and femoral artery were determined with ELISA (provided by the Department of Immunology, the Third Military Medical University). ET in portal vein was assayed by a quantitative azostromatic coloration limulus test (kit provided by the Shanghai Medical Analysis Institute).

RESULTS

Change of serum TNFα levels (Table 1 and Figure 1)

Table 1 TNFα level ($\bar{x}\pm s_{\bar{x}}$, pg/L) of rats 2h and 6h after operation

Groups	Time(h)	PV	HV	RHU(%)	FA
Control	2	54.83±3.24			
	6	52.14±1.87			
AHNP	2	182.48±9.90	119.85±5.64	38.63	127.32±7.15
	6	354.91±25.50	280.59±20.02	20.95	310.82±7.97

PV: portal vein; HV: hepatic vein; RHU: rate of hepatic uptake; FA: femoral artery.

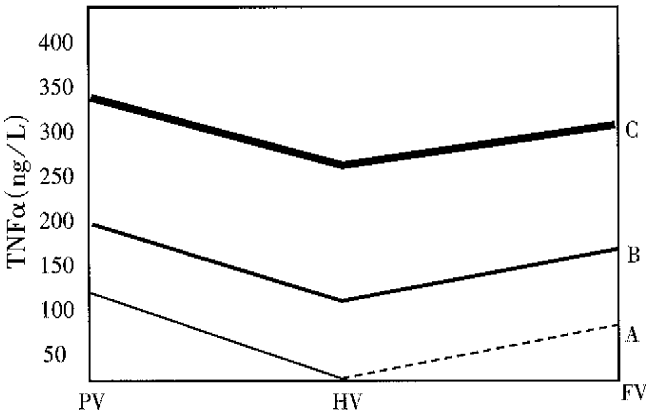

Figure 1 Changes of TNFα level in portal vein, hepatic vein and femoral artery 2 and 6 h after operation. A: TNFα level of control group 6 h after operation; B,C: TNFα level of AHNP group 2 and 6 h after operation.

There was significant difference in TNFα levels among the samples from portal vein, hepatic vein and femoral artery (P<0.05) 2 and 6 hours after operation, and the difference was also significant as compared with the control group (P<0.05). Two hours after operation, TNFα level in hepatic vein and femoral artery was lower than in portal vein (P < 0.05), but no difference was observed between that in femoral artery and hepatic vein. Six hours after operation, TNFα level in femoral artery was obviously higher than in hepatic vein (P<0.05), but remained lower than in portal vein (P<0.05). There was significant difference in the rate of hepatic uptake of TNFα between AHNP group and control group (P<0.05).

Change of plasma ET level (Table 2)

Table 2 Change of plasma ET level ($\bar{x}\pm s_{\bar{x}}$, EU/ml)

Groups	Time(h)	Plasma ET
Control	2	0.022±0.007
	6	0.033±0.006
AHNP	2	0.028±0.002
	6	0.340±0.038

Two and 6 hours after operation, there was significant difference in plasma ET level in portal vein of AHNP rats. When compared with the control group,significant difference of plasma ET level in portal vein of AHNP rats was observed at 6 hours, but not at 2 hours after operation.

DISCUSSION

TNFα is an important inflammatory mediator during the development of AHNP. It can damage the pancreas and ex-pancreatic vital organ through the following mechanism: ① Enhancing the adhesion of PMN to endothelium. Recent research shows that there are some adhesive molecules or receptors, such as ICAM-1, ELAM-1 and VCAM-1 on the surface endothelia. TNFα can stimulate the expression of receptors for adhesive molecules on the surface of endothelium, leading to the adhesion of unstimulated PMN to endothelia and the migration of the PMN to inflammatory loci through vascular barrier. The interaction of PMN with target tissue cells and the releasing of oxygen free radicals (OFR) and neutral proteinase could cause the damage of adjacent tissues. ② The cytotoxic effect of TNFα. TNFα can bind with the specific receptors on the cellular membrane and facilitate the production of OFR, which damages the cell membrane and DNA, and cleaves DNA into fragments by endonuclease, leading to the death of cells. ③ TNFα can activate the inflammatory cells, stimulate the release of PMN from bone marrow, and promote the degranulation, the production of OFR and the releasing of lysosome enzyme, alkali proteinase, etc. from eosinocytes. The persistent increase of plasma TNFα may promote the endocytosis, degranulation and the releasing of OFR and leukocyte enzymes (lysosome enzyme, plastic enzyme, etc.), resulting in the continuous hemorrhage, necrosis of pancreas and the development of MOF.

The exact mechanism of production, the production and absorption site of TNFα during the progression of AHNP remained unknown[2]. The stimulation of inflammatory cells by pancreatic enzyme was believed to be related with the production of TNFα. It is indicated that the disturbance of microcirculation of pancreas may lead to release of trypsinogen activated peptides[3], suggesting that the disturbance of microcirculation of pancreas may be the primary factor responsible forproduction of TNFα. Our results revealed that, at the early stage of AHNP, the TNFα level in portal vein was elevated obviously, which was significantly higher than in hepatic vein and femoral artery. There was no difference in TNFα level between hepatic vein and femoral artery. So it is concluded that at the early stage of AHNP, TNFα in portal vein may originate from pancreas, spleen and gastrointestinal tract, and the liver may clear some of TNFα originated from portal vein. With the aggravation of AHNP,

TNFα in portal vein increased rapidly, and so did that in femoral artery, both being higher than in hepatic vein, but still lower than in portal vein. Based on these results, we believe that lung may be the organ of secondary production site of TNFα. TNFα in hepatic vein mainly originate from pancreas, spleen and gastrointestinal tract, and part of TNFα in hepatic vein might come from the liver. Although the liver could clear some TNFα, its potential is somewhat limited. As the hepatic damage gets worse, its clearance to TNFα decreases.

TNFα is a soluble polypeptide with an MW of 17Ku, under the condition of severe infection, macrophagocytes, T cells and NK cells would produce TNFα due to the action of Gram negative bacteria and lipopolysaccharide. It is believed that at early stage of AHNP, the production of TNFα is not associated with ET[4]. Our results showed that plasma TNFα level elevated significantly when there was no increase of ET level in portal vein, indicating that ET was not the primary factor for the production of TNFα in AHNP rats, but TNFα level elevated much more significantly with the increase of ET. Recent studies also showed that, in late-stage AHNP, ET was contributed to the substantially increased production of TNFα. The synergetic effect of TNFα and ET is an important cause of high morbidity and mortality of AHNP. Therefore, control of infection, inhibition of the TNFα production, and removal of secreted TNFα are of great importance for the treatment of AHNP.

REFERENCES

1 Hughes CB, Grewal HP, Gaber LP, Kotb M, El-din M, Mann M *et al.* Anti-TNFα therapy improves survival and ameliorates the pathophysiologic sequelae in acute pancreatitis in the rat.*Am J Surg*, 1994;171(1):274-278
2 Grewal HP, Mohy EL, Din A, Kotb M. Induction of tumor necrosis factor in severe acute pancreatitis and its subsequent reduction after hepatic passage. *Surgery*,1994;115(1):213-221
3 Mothofer K, Castillo CFD, Frich TW, Foitzik T, Bassi DG, Lewandrowski KB, *et al*.Increased intrapancreatic trypsinogen active ischemia induced experimental pancreatitis. *Ann Surg*,1995;221(4):364-371
4 Hughes CL, Gaber LW, Kotb M, Mohey el din AB, Pabst M, Gaber O. Induction of acute pancreatitis in germ free rats: evidence of a primary role for tumor necrosis factor alpha. *Surgery*,1995;117(1):201-205

A clinical evaluation of serological diagnosis for pancreatic cancer *

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Subject headings pancreatic neoplasms/diagnosis; tumor markers, biological; antigens, neoplasm/analysis; CA-19-9 antigen/analysis; pancreatopeptidase/analysis; carcinoembryonic antigen/analysis; alpha 1-antitrypsin/analysis; enzyme-linked immunosorbent assay; radioimmunoassay

Abstract

AIM To assess the diagnostic values of tumor markers for pancreatic cancer.

METHODS Pancreatic cancer-associated antigen from colonic mucosa (PCAAc), pancreas-specific antigen (PaA), pancreatic oncofetal antigen (POA) and minimolecular pancreatic antigen (mPOA) were detected by double antibodies Sandwich ELISA; CA19-9, elastase 1 (E1), human pancreatic elastase 1 (HPE1) and carcinoembryonic antigen (CEA) by radioimmunoassay (RIA); general activities of ribo-nuclease (RNase) and its isoenzymes (RNase I and RNase II) by biochemistry and PAEG; glycylproline dipeptidyl aminopeptidase (GPDA) by biochemistry and α1-antitrypsin (α1AT) by rocket immunoelectrophoresis (rocket-IE).

RESULTS The detection of serum POA, mPOA, PaA, PCAAc, CA19-9, RNase and RNase I was able to differentiate pancreatic cancer from the benign disorders and non-pancreatic malignancies with a sensitivity from 66.75% to 80.0% and a specificity from 88.5% to 96.69%. POA, mPOA, PCAAc, HPE1, E1 and GPDA were related to the pancreatic cancer at the head which demonstrated higher sensitivity from 63.64% to 85.71%. The detection of serum HPE1 was especially helpful for the diagnosis of pancreatic cancer with smaller diameters. The determination of 3 or 4 kinds of tumor markers simultaneously would increase the detection rate of pancreatic cancer, which will be an important procedure for the diagnosis of this malignancy.a¤

CONCLUSION A single test of tumor markers is helpful to detect pancreatic cancerclinically,but the determination of 3 or 4 kinds of tumor markers simultaneously would significantly increase the detection rate of pancreatic cancer, which will be an important procedure for the diagnosis of this malignancy.

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INTRODUCTION

The incidence of pancreatic cancer is increasing yearly, but its early diagnosis remains very difficult, for the symptoms are faint, vague and nonspecific. Therefore, it is important to seek a tumor marker with satisfactory specificity and/or sensitivity for the diagnosis of the cancer clinically. This paper reports our ten years' research on tumor markers to evaluate the clinical application of the serum detection of tumor markers for the diagnosis of pancreatic cancer.

MATERIALS AND METHODS

Samples

We detected 13 tumor markers in samples from 763 cases including 143 pancreatic cancer, 125 liver or peri-ampullar carcinoma and 120 gastrointestinal carcinoma, which were identified by surgical operation and/or pathological examination. One hundred and five cases of acute and chronic pancreatitis were observed. The acute cases were identified by clinical approach and chronic ones by X-ray photograph, surgical operation and pathological examination. Blood samples from 270 healthy fasting donors were examined likewise and served as control. Fasting blood samples were obtained through venous puncture in the morning and the serum was preserved at -20℃ for assay.

Methods

Pancreatic cancer-associated antigen from colon mucosa (PCAAc), pancreas-specific antigen (PaA), pancreatic oncofetal antigen (POA) and minimolecular pancreatic antigen (mPOA) was detected by double antibodies Sandwich ELISA[1-4]; CA19-9, elastase 1 (E1), human pancreatic elastase 1 (HPE1) and carcinoembryonic antigen (CEA) by radioimmunoassay (RIA); general activities of ribonuclease (RNase) and its isoenzymes (RNase I and RNase II) by biochemistry and PAEG[5]; glycylproline dipeptidyl aminopeptidase (GPDA) by biochemistry according to Kojima's report[6] and α1-antitrypsin (α1AT) by rocket immunoelectrophoresis (rocket-IE).

Statistical analysis

The data of distribution percentage, *t* test, FLSD test, stepwise regression and discriminatory analysis of the tumor markers were processed on a microcomputer with a medical program system, SPMR, designed by the department of mathematics of our college.

RESULTS

Single assay of the tumor markers

Circulating levels of the tumor markers in patients with pancreatic cancer as well as those with benign pancreatic diseases, non-pancreatic malignancies and normal control subjects are shown in Tables 1 and 2. All the tumor markers except for RNase 2 and CEA were elevated in the sera of patients with pancreatic cancer and exhibited a sensitivity ranging from 45% to 80.8% and a specificity from 66.67% to 97.14%.

Tumor markers related to location, size and differentiation of pancreatic cancer

As shown in Table 3, the detection of POA, mPOA, PCAAc, E1, HPE1 and GPDA demonstrated that the positive percentage ranged from 63.64% to 85.73% in the patients with pancreatic cancer at the head, which was significantly higher than that at the body or tail and entire. The detection of PaA and CA19-9 could be better for the cancer at tail and/or body. It is worth-mentioning that higher positivity of the detection with HPE1 was found in the patients with the tumor smaller than 5.0 cm. The elevation of serum mPOA, PCAAc, PaA, HPE1, E1 and GPDA was closely related to well-differentiated cancer.

Simultaneous detection of tumor markers

Although single assay of tumor markers distinguishes pancreatic cancer from the other groups, the conjoint detection could improve the diagnostic positivity and specificity for the cancer clinically. As shown in Table 4, four tumor markers, including PCAAc, mPOA, PaA and GPDA were selected for this purpose, and the results showed a positivity of 40.6%-80.8% for the diagnosis of pancreatic cancer by single test, 75%-96.77% by conjoint detection with any two markers and 100% by any three markers.

Table 1 Sensitivity and specificity of a single test of tumor markers for pancreatic cancer

Groups	Sensitivity (%)	Specificity (%)
POA	67.80	88.50
mPOA	74.36	95.6
PCAAc	68.70	96.49
PaA	78.79	89.40
HPE1	66.67	83.25
E1	62.50	66.67
CA19-9	70.97	90.63
GPDA	42.12	68.42
RNase	80.80	80.00
RNase I	45.00	94.28
RNase II	25.00	97.14
α1AT	50.00	74.28
CEA	25.00	85.71

Table 2 Results of single test of tumor markers

Groups	Normal control	Pancreatic cancer	Non-pancreaticmali gnancy	Pancreatitis
POA(U/ml)	84.80±13.96(1/40)	252.5±164.33(19/28)	136.37±93.63(9/62)	101.98±57.88(1/15)
mPOA(mg/L)	4.88±1.96(2/40)	18.45±10.57(29/39)	6.24±3.82(5/62)	4.69±1.52(0/17)
PCAAc(mg/L)	12.59±6.34(1/40)	57.25±82.93(23/33)	12.64±5.14(3/49)	13.53±9.91(0/14)
PaA(μg/L)	13.05±3.84(3/64)	61.54±45.59(26/33)	17.62±10.31(5/44)	21.35±11.83(3/20)
HPE1(μg/L)	23.80±3.40(1/82)	83.30±73.40(62/93)	48.93±54.63(26/63)	124.90±168.30(25/35)
E1(ng/dl)	276.60±85.36(0/10)	1116.00±1281.00(10/16)	1321.00±2842.00(9/13)	
CA19-9(U/L)	7.90±9.74(2/81)	165.40±128.03(19/31)	139.88±121.72(6/15)	
GPDA(U/L)	54.84±10.80(0/60)	101.02±75.84(14/33)	64.89±72.64(27/69)	50.94±15.50(2/25)
RNase(U/ml)	58.19±11.09(1/30)	92.22±19.66(16/20)	65.88±12.32(5/20)	67.95±9.77(2/15)
RNase I(U/ml)	9.53±13.43(1/30)	39.09±39.31(9/20)	9.23±8.98(1/20)	11.87±11.30(1/15)
RNase II(U/ml)	50.75±13.36(0/30)	52.78±29.68(5/20)	56.89±16.82(0/20)	60.17±19.55(1/15)
α1AT(mg/L)	30.51±6.35(1/30)	49.86±18.47(10/20)	38.65±16.30(6/20)	39.23±10.02(3/15)
CEA(mg/L)	11.15±2.95(1/20)	16.82±10.26(5/20)	15.73±11.98(5/20)	10.22±3.42(0/15)

Table 3 The relationship between the tests of tumor markers and the location, size or differentiation of pancreatic cancer

Groups	Location			Size			Differentiation	
	Head	Body or tail	Entire	<5.0 cm	5 cm-9.0 cm	<9.0cm	Well & moderate	Poor & un-differentiated
POA	9/11(81.82)	3/6(50.00)	1/3(33.33)	4/8(50.00)	8/10(80.00)	1/2(50.00)		
mPOA	22/27(81.48)	5/9(55.56)	2/3(66.67)	5/5(100.00)	14/19(73.68)	10/15(66.67)	10/10(100.00)	9/13(69.23)
PCAAc	17/22(77.27)	4/9(44.44)	1/2(50.55)	3/4(75.00)	9/13(69.23)	3/6(50.00)	5/5(100.00)	2/4(50.00)
PaA	16/22(72.73)	10/11(90.91)	2/2(100.00)	4/4(100.00)	12/15(80.00)	6/10(60.00)	8/8(100.00)	4/5(80.00)
E1	14/17(82.35)	2/6(33.33)	2/3(66.67)	6/8(75.00)	4/10(40.00)	1/2(50.00)	3/6 (50.00)	2/4(50.00)
HPE1	35/41(85.37)	20/35(57.14)	7/17(41.18)	40/51(78.43)	15/25(60.00)	7/17(41.18)	43/60(71.67)	19/33(57.58)
GPDA	14/22(63.64)	0/9(0.00)	0/2 (0.00)	8/17(47.06)	3/7(42.86)	3/7(42.86)	4/7 (57.14)	1/4(25.00)
RNase	31/41(75.61)	24/35(68.23)	15/17(88.23)	20/29(68.96)	19/25(76.00)	15/17(88.24)	46/ 60(76.67)	24/33(72.72)
CA19-9	10/17(58.82)	6/8(75.00)	6/6(100.00)	7/15(46.67)	9/12(75.00)	4/4(100.00)	8/9(88.89)	8/8(100.00)

Table 4 Results of simultaneous detection of four tumor markers

Groups	Pancreatic cancer (n=30)	Non-pancreatic malignancy (n=43)	Benign diseases (n=21)
All the tests negative	0(0.00)	17(39.54)	18(85.71)
Single test positive	4(12.90)	21(48.83)	3(14.29)
Double tests positive	6(19.36)	5(11.63)	0(0.00)
Three tests positive	16(51.61)	0(0.00)	0(0.00)
All the tests positive	5(16.13)	0(0.00)	0(0.00)

DISCUSSION

Single assay significance of tumor markers for pancreatic cancer diagnosis

A single assay of tumor markers was helpful in distinguishing patients with pancreatic cancer from normal controls and those with non-pancreatic malignancy. By stepwise discriminatory analysis, seven out of all 13 tumor markers including CA19-9, POA, mPOA, PaA, PCAAc, RNase and RNase I were associated well with the diagnosis of pancreatic cancer with the sensitivities ranging from 45% to 80.8% and the specificity from 80% to 96.49%. Although the detection of HPE1 and E1 had the sensitivities of 66.67% and 62.5% to the disease, these were regarded as helpful diagnostic indices for the poor differentiation value. A single test of GPDA, α1AT, CEA and RNase II was not significant to the diagnosis of the cancer for their unsatisfied sensitivity and/or specificity.

Although the mechanism of the tumor markers expressed in blood was not clear, the problem of false positive rate was noticed in clinic; especially to some carcinoma adjacent to pancreatic cancer such as primary hepatic carcinoma, cholangioampulla carcinoma, etc.[7,8]. In our study, the false positive rates ranging from 8.33% to 21.74% was found in patients with primary hepatic carcinoma or cholangioampulla carcinoma with a single test of POA, mPOA, PCAAc, PaA and RNase I, and 36.92% to 87.09% with CA19-9, HPE1, E1 and GPDA. These suggest that a single test of tumor markers has limitations for making a definite diagnosis of pancreatic cancer unless it is combined with other detection, especially the image analysis.

Correlation of tumor markers to location, size or differentiation of cancer

The higher positive rates in detecting POA, PCAAc, HPE1, E1 or GPDA were found in patients with pancreatic head cancer, with the sensitivities of 63.64%-85.71%. It is interesting that GPDA were only elevated in the sera of patients with the cancer at pancreatic head but not at body or/and tail. The mechanism of elevated GPDA might be related to the obstruction of bile duct. Unfortunately, none of the 13 tumor markers were significantly related to the cancer at body or/and tail.

According to our studies, the sensitivities of CA19-9, RNase, GPDA and POA detection were gradually increased with the development of the mass, which were 46.67%-50% in the patients with the tumor size less than 5.0cm. Although elevated serum mPOA and PCAAc were associated with the patients with the tumor size less than 5.0cm, the methods per se for correct diagnosis of pancreatic cancer in the early stage still need further studies. It is worth-mentioning that the detection of serum HPE1 was significantly related to the tumors less than 5.0cm. In this study, significantly higher serum HPE1 was observed in 22 of 51 cases with the tumor size less than 3.0cm, among them the concentration was 118.1 μg/L ± 102.7 μg/L and the positive rate was 78.46%. Furthermore, 4 of 6 cases with the tumor size less than 2.0cm also had elevated serum HPE1. This suggests that serum HPE1 might be a reference tumor marker helpful for the diagnosis of patients in the early stage.

Correlation of tumor markers with differentiation of cancer

The serum variation of some tumor markers such as mPOA, PCAAc, PaA, HPE1 and GPDA were related to the differentiation degree of the tumors. Our results showed that positive rate and elevation range in highly and moderately differentiated pancreatic cancer were higher than low and non-differentiated one, which suggested that these tumor markers can be regarded as differentiating antigen for assessment of the transformation of the disease.

Significance of combined assay of tumor markers for pancreatic cancer

Combined detection of tumor markers would increase and improve the detection rate and the diagnostic accuracy of the malignancy. According to our data, the sensitivity to the diagnosis of the disease was 25%-80.0% with single test of the 13 tumor markers, 75%-96.77% with two tests, and 100% with over 3 tests. The combined tests with simultaneous positive results of 2 out of the 13 tumor markers could be used to rule out pancreatic benign disease. Non-pancreatic malignancies never showed positive results of over three tumor markers simultaneously. Our results suggest that determination of 3 or 4 tumor markers conjointly would increase the detection rate of pancreatic cancer, especially for screening among large populations. It might become an important procedure for the diagnosis of this malignancy.

REFERENCES

1 Guo XZ, Yu SY, Guo P, Dai XJ, Liu WW. The value of measurement of serum pancreatic oncofetal antigen in the diagnosis of pancreatic carcinoma. *Chin J Dig*,1989;9(2):88-91

2 Zhao XY, Yu SY, Guo P, Bai L. Preliminary study of the diagnosis of pancreatic cancer with a pancreatic associated antigen.*J Med Coll PLA*, 1995;10(3):162-165

3 Zhao XY, Yu SY, Bai L, Guo P. Diagnosis of pancreatic cancer with simultaneous detection of a pancreatic cancer-associated antigen and a pancreas-specific antigen.*Chin J Intern Med*,1990;29(10);612-615

4 Dai XJ, Yu SY, Guo P, Guo XZ. A preliminary study on serum pancreatic elastase I for the diagnosis of pancreatic cancer. *Chin J Dig*, 1989;9(5):299-300

5 Wang YM, Yu SY, Guo P. A preliminary study on serum ribonuclease and isoenzyme for the diagnosis of pancreatic cancer. *Chin J Dig*, 1988:8(3):134-137

6 Kojima J, Kanatani M, Nakamura N, Kashiwagi T, Tohjoh F, Akiyama M. Serum and liver glycylpronine dipeptidyl amino peptidase activity in rats with experimental hepatic cancer. *Clin Chin Acta*,1980;107(1-2):105-110

7 Tian T, Appert H, Myles J, Howard JM. Prognosis value of serum CA19-9 levels in pancreatic adenocarcinoma.*Ann Surg*,1992;215(4):350-355

8 Saito S, Taguchi K, Nishimuri N, Watanabe A, Ogoshi K, Niva M et al. Clinical usefulness of computer associated diagnosis using combination assay of tumor markers for pancreatic carcinoma.*Cancer*, 1993;72(2):381-388

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ERCP and CT diagnosis of pancreas divisum and its relation to etiology of chronic pancreatitis *

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Subject headings pancreatitis/etiology; pancreas divisum/radiography; cholangiopancreatography, endoscopic retrograde; pancreatitis/diagnosis; chronic diseases; amylases/blood; pancreatic ducts/radiography

Abstract

AIM To inquire into the ERCP and CT features of pancreas divisum (PD) and its role in the etiology of chronic pancreatitis.

METHODS Fourteen patients with PD were analyzed in regard to the findings in ERCP and CT, the activities of serum amylase and the incidence of pancreatitis. Dorsal ductography via minor papilla cannulation was performed in six of them.

RESULTS The length of dorsal and ventral pancreatic duct was 16.56cm ± 2.52cm and 5.55cm ± 1.46cm. Most of the patients had dilatation of dorsal (10/14) and ventral (8/14) duct and the stenosis of dorsal duct terminal (10/14). Delayed clearance of contrast in dorsal duct was found in 8 patients. The size and contour of the pancreas were normal in all the patients at conventional CT. Pancreatitis was identified in 13 patients.

CONCLUSION Dorsal ductography was necessary in the diagnosis of PD. Conventional CT play little role in the diagnosis of PD. Patients with PD run a higher risk of pancreatitis due to the stenosis of the minor papilla.

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INTRODUCTION

Pancreas divisum (PD) is a congenital pancreatic ductal anomaly in which the dorsal and ventral pancreatic ducts fail to fuse (complete PD, CPD) or the rudimentary ducts communicate through a fine secondary or tertiary branch (incomplete PD, IPD) during the organogenesis[1-4]. It is rare in Asia[1]. There has been a heated contraversy between those whose epidemicologic studies showed increased prevalence of pancreas divisum among patients with idiopathic pancreatitis and those who found no significant difference in their control population[1]. We analysed the ERCP and CT manifestations of PD in 14 patients and inquired into its possible role in the etiology of chronic pancreatitis.

MATERIALS AND METHODS

Between 1983 and 1994, endoscopic retrograde cholangiopancreatography (ERCP) was performed in 3554 patients suspected to have pancreaticobiliary diseases. Among them, the diagnosis of IPD was made in 8 patients when a typical ventral pancreatic duct was first observed,with further filling, a dominant dorsal ductal system was filled via a fine secondary or tertiary branch (Figure 1). On the other hand, dorsal ductography via minor papilla cannulation was attempted in 10 cases when the "main pancreatic duct" was short and tapered, without communicating with pancreatic body and tail (Figure 2). An ideal dorsal ductogram was obtained in 6 patients, and the body and tail segmental duct was filled only via the minor papilla (Figure 3). CPD was confirmed in these patients.

The clearance time of the contrast in the dorsal and ventral duct was recorded in all patients. If the contrast was present at 12min after the duct had been filled in the prone position, the patient was considered to have a prolonged drainage.

This series consisted of 4 men and 10 women. The mean age was 46.1 years ± 12.2 years (24-69). Six with IPD and five with CPD complained of chronic upper abdominal pain, the others had jaundice, which was caused by biliary stones or biliary duct carcinoma confirmed by CT or pathologic examinations.

CT examination was made in all the patients, with a Shimadzu 3000TX or Somatom DR3 scanner. Collimation of 5 mm and 5 mm or 10 mm scan spacing were used.

Serum amylase activity was assayed using blue

starch method in all patients before ERCP was performed.

We studied the ERCP and CT features of pancreas and the activity of serum amylase and analysed the relationship among the sphincter motility, the dilated degree and drainage time of the ducts and the incidence of pancreatitis.

RESULTS

Ventral and dorsal ductograms

Dorsal duct. The mean length was 16.56cm±2.52 cm. The moderate dilatation and irregular contour were seen in 10 cases (associated with protein emboli in 2) and the others had a normal shape.

Ventral duct. The length was 5.55 cm±1.46 cm and dilatation existed in 8 patients (associated with protein emboli in 2 patients). No anomalous changes were found in the remaining 6 cases.

Communicating duct. Its shape was similar to that of pancreatic ductal secondary or tertiary branch and the contour was regular (Figure 1). The united point of the branch with the dorsal duct was situated in the pancreatic neck segment and the angle was 42.7° ±19.2° .

The trend and drainage time. The results are shown in Table 1.

Table 1 The trend and drainage time of dorsal (ventral) duct

Group	Trend			Drainage time	
	Ascend	'S' like	Descent	>12min	<12min
IPD	3(8)	2(0)	3(0)	4(1)	4(7)
CPD	3(5)	2(0)	1(1)	4(0)	2(6)

Sphincter motility of dorsal duct

The rigidity and contriction of the ductal sphincter segment were present in nine of ten patients with dorsal ductal dilatation and one of four with normal shape (Figure 4).

CT manifestations

All patients had a normal pancreatic size and contour. Among them, the dorsal ductal dilatation was found in one patient with IPD and two with CPD who also had apparent dilatation at ERCP.

Serum amylase activities

The activities were significantly increased in two of IPDs and three of CPDs (585IU/L-1553IU/L) and were normal (60 IU/L - 180 IU/L) in the others.

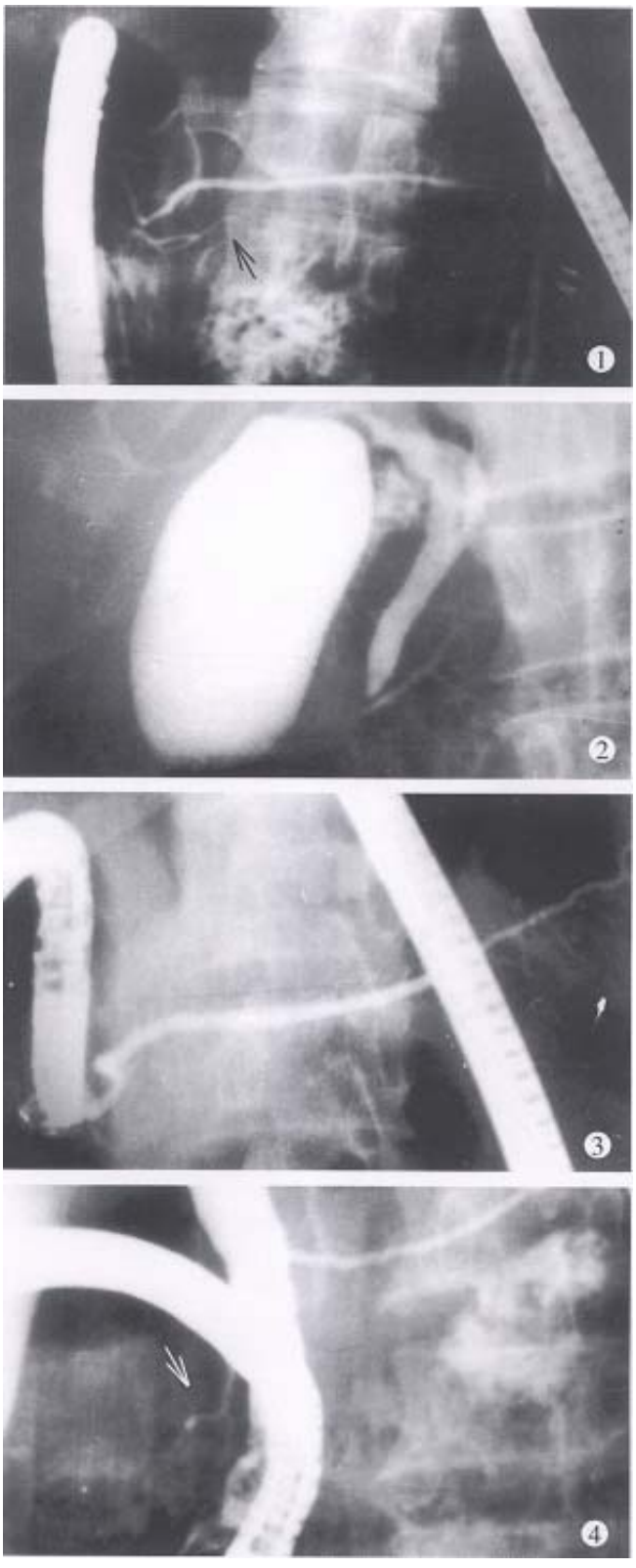

Figure 1 The ERCP manifestations of IPD (↑ : communicating branch).

Figure 2 The ventral ductograms of CPD at conventional ERCP. The duct was short, tapered and arborized into a fine side branches without communicating with the body and tail of the pancreas.

Figure 3 The dorsal ductograms of the above case via the minor papilla cannulation. The duct was dominant and trended from the pancreatic head to tail.

Figure 4 A permanent stenosis of the dorsal ductal terminal was present at the ductograms via the minor papilla cannulation (↑).

Pancreatitis and biliary tract diseases

Pancreatitis was found in seven of IPDs and six of CPDs according to the diagnostic standards of Guilin Symposium in 1987. The bile duct stones were found in 1 of IPDs and 3 of CPDs. Two patients had carcinoma of the biliary duct.

DISCUSSION

Because the conventional ERCP fails to show the whole pancreatic ductal system. Dorsal pancreatic ductography should be applied to those patients who are suspected to have PD. Minor papilla cannulation can be achieved using the catheters with tapered or 25-gauge needle tips. If the minor papilla or its orifice is not distinctly seen, secretin can be administered at a dose of 1 IU/kg body weight to stimulate the pancreatic juice to flow out.

At conventional ERCP, when a characteristic ventral duct was seen, and with further filling, it was communicated with a dominant dorsal duct system through a fine secondary or tertiary branch, IPD could be diagnosed. If no communicating branch was found and the dorsal duct was not filled or clear secretions flowed briskly from the minor papilla instead of the major papilla in response to secretin administered, IPD or CPD could be suspected and dorsal ductography via the minor papilla cannulation should be performed. Under this condition, if the dorsal duct was dominant and trended from pancreatic head to tail, the diagnosis of CPD could be made.

The dorsal and ventral ducts might have some other abnormal changes, including dilatation, distortion, contour irregularities and protein emboli. Anomalous pancreaticobiliary ductal union was found in 2 patients in this study. It implied that the two kinds of pancreatic duct adnormalities were likely to have a common origin in the embryogenesis.

No characteristic manifestations were found in conventional CT. It suggested the limitation of conventional CT to the diagnosis of PD. Lindstrom *et al*[2] investigated the pancreatic size and contour in 60 patients undergoing the thin-section CT and confirmed that the cranio-caudal length of the pancreatic head in CPD was longer by about 40% than that in normal subjects. He thought that this might indicate the presence of PD, but a local mass must be ruled out. Zeman *et al*[3] claimed that a characteristic oblique fat cleft in the pancreas head separating the ventral from dorsal pancreas could be seen in 25% of patients at thin section CT.

In the patients with PD, most of the pancreatic juices excreted via the relatively strictured minor papilla. Though the juices could fully drain in the basal stage of secretion, it could be hindered in the peak stage or when the minor papilla was injured by the mechanical, chemical and biological factors. As a result, the attack of pancreatitis was not surprised. In this study, the incidence of pancreatitis in the patients with IPD (87.5%) and CPD (100%) was significantly higher than that in the reference patients with other pancreaticobiliary diseases (15.3%) which was previously reported by us[4]. It indicated that PD was likely to have an etiologic effect on pancreatitis. Besides, a permanent stenosis of the dorsal ductal terminal and the prolongation of the ductal drainage time were present in most of the patients, especially in the patients associated with pancreatic duct dilatation. Therefore there might be a relationship between the attack of pancreatitis and the limitation of the pancreatic juices drainage.

REFERENCES

1 Warshaw AL, Simeone JF, Scapiro RH, Warshaw NF. Evaluation and treatment of the dominant dorsal duct syndroma (pancreas divisum redefined). *Am J Surg*, 1990;159(1):59-66
2 Lindstrom E, Ihse I. Computed tomography findings in pancreas divisum.*Acta Radiol Diagnosis*,1989;30(6):609-613
3 Zeman RK, McVay LV, Silverman PM, Cattau EL, Benjamin SB, Fleischer DF *et al*.Pancreas divisum: thin section CT.*Radiology*, 1988;169(2):395-398
4 Wu BH, Lu WF. The association between anomalous pancreaticobiliary duct union and pancreaticobiliary diseases.*Chin J Radiol*,1993;27(12):840-845

Pathogenetic factors affecting gastroesophageal reflux in patients with esophagitis and concomitant duodenal ulcer: a multivariate analysis

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Subject headings esophagitis/etiology; gastric acid/secretion; duodenal ulcer/etiology ; gastroesophageal reflux/etiology; hydrogen-ion concentration; multivariate analysis

Abstract

AIM To assess the relationship between gastric acid output (GAO) and both pattern of gastroesophageal reflux (GER) and esophageal lesions, and to evaluate the role of GAO and other potential pathogenetic factors in the development of esophagitis.

METHODS Gastric acid secretory testing and 24-h intraesophageal pH monitoring were performed in 31 patients with esophagitis and concomitant duodenal ulcer (E+DU) and compared with those of 72 patients with esophagitis (E) alone.

RESULTS The GAO in patients with E+DU was significantly higher than in patients with E ($P<0.05$). There was no significant difference between the two groups of patients as to endoscopicl findings and parameters of GER ($P>0.05$). A multiple regression analysis with stepwise deletion showed that the pre-sence of hiatal hernia (HH), GER in upright position and age appeared to correlate significantly with the presence of esophagitis.

CONCLUSIONS No parallel relationship between GAO and severity of GER or esophageal lesions exists in patients with E+DU, and that GAO is not a major pathogenetic factor in GER disease.

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INTRODUCTION

It is generally agreed that gastroesophageal reflux (GER) may be multifactorial in its pathogenesis. Over the past decade, many investigators have focused their attention on the role of lower esophageal sphincter (LES), hiatal hernia (HH), esophageal mucosal sensitivity, and esophageal or gastric emptying in the development of GER disease. Some studies have shown that LES, hiatal hernia, esophageal emptying and esophageal mucosal sensitivity are, to a certain extent, involved in the pathogenesis of GER[1-10]. However, although gastric acid is believed to be an important factor in the development of GER disease, it is still unclear whether the severity of GER and esophageal lesions is necessarily related to increased gastric acid output. Moreover, little is known which factors are crucial in the pathogenesis of GER disease. Therefore, the aims of this study are: to assess the relationship between gastric acid output (GAO) and both pattern of GER and severity of esophageal lesions in patients with esophagitis and concomitant duodenal ulcer (E+DU), in comparison with patients with esophagitis only (E); and to evaluate the role of GAO and other potential pathogenetic factors in the development of reflux esophagitis by multiple regression analysis with stepwise deletion.

PATIENTS AND METHODS

Patients

Two groups of patients were enrolled in this study. Of these patients, 31 (27 men, 4 women; mean age of 44 years) had E+DU and 72 (53 men, 19 women; mean age of 42 years) had esophagitis only. All patients had symptoms of GER disease, i. e., chronic heartburn and regurgitation, and/or upper abdominal pain, for a median duration of 55.4 months (range from 3 to 120 months). None of the patients had taken H2-receptor blockers or H^+/K^+-ATPase inhibitors for 2 weeks before 24-h pH-monitoring and gastric acid secretory testing.

Methods

All patients underwent upper GI endoscopy, followed by 24h intraesophageal pH monitoring and acid secretory testing over a 2-week period. Endoscopy was performed by the same gastroenterologist in all patients. The degree of esophagitis was

assessed endoscopically by using the criteria of Savary and Miller[11] (grades I to IV). Of these patients, only few presented grades II and III esophagitis, and therefore grades I and II, and III and IV, respectively, were grouped together.

The 24-h intraesophageal pH monitoring was carried out by a routine method used in our laboratory[12,13]. A glass pH-electrode with an incorporated potassium chloride reference (Ingold electrode, No. 440) was introduced via the nasoesophageal route and positioned with the tip 5 cm above the gastroesophageal junction, identified with the pH[a2]meter. Esophageal pH values were recorded with a solid-state recorder (Autronicord CM 18).

Analysis of pH recording was made on a computer with a dedicated program. The parameters recorded included the frequency and duration of GER in upright position (day-time) and supine position (night-time), and GER frequency exceeding 5 minutes. GER was defined as abnormal if total reflux duration amounted to 7% during the 24-h monitoring[14].

Basal acid secretory analysis was performed in the absence of any antisecretory medication for 2 weeks before the study, in accordance with Raufman *et al*[15]. In brief, after a nasogastric tube was introduced into and positioned in the gastric antrum, the gastric contents were emptied by aspiration. Four consecutive 15-min samples of gastric secretion were obtained by continuous aspiration, and the samples were titrated with 0.01 N NaOH to pH 7.0. Fasting basal acid output (BAO) was expressed as milliequivalents of acid per hour(mEq/h). After a BAO was obtained, the patients underwent stimulated acid secretory testing. Six μg/kg of pentagastrin were injected intramuscularly. Sum of the four highest consecutive 15-min samples was represented as a maximal acid output (MAO), and that of the two highest consecutive 15-min period within 2 h of receiving the stimulant was represented as a peak acid output (PAO). At the same time, basal gastrin level in the serum was measured. Normal values for BAO, MAO, PAO and basal gastrin in our laboratory are as follows: BAO, 0.37 mEq/h ± 0.27 mEq/h; MAO, 2.05 mEq/h ± 1.07 mEq/h; PAO, 3.36 mEq/h ± 1.19 mEq/h; and basal gastrin, 37.8 ng/L ± 2.84ng/L.

Chisquare test and Student's ^{t} test were used to evaluate the data of GI endoscopy and gastric acid secretion, respectively. Statistical evaluation was made using the Mann-Whitney *U* test for the parameters of 24 h intraesophageal pH monitoring. Multiple regression analysis with stepwise deletion was used to evaluate role of some potential pathogenetic factors in the development of reflux esophagitis, which were rated in order of importance. The methods and steps are as follows:

a. The formula of multiple regression:

$$y = b0+b1X1+b2X2+\cdots+bkXk$$

where y and b0 represent a dependent variable and a constant factor, X1, X2, ⋯, Xk represent independent variables, and b1, b2, ⋯, bk are the standard partial regression coefficient of independent variables[16].

b. Dependent variable and independent variables. In this study, the dependent and independent variables were selected on the basis of the hypothesis that the pathogenesis of the reflux esophagitis was multifactorial, and GER, gastric acid, hiatal hernia, etc. may be all involved, to a certain extent, in the pathogenesis of esophagitis. Accordingly, esophagitis was defined as the dependent variable, and its numerical values assigned to different grades of esophagitis were: 1 (Grade I or II) and 2 (Grade III or IV). Ten variables such as age, HH, GER, gastric acid, etc. were de fined as the independent variables. Table 1 gives in detail these independent variables and their definition.

Table 1 Independent variables

Variables	Items	Definition
X1	Age	Years
X2	Hiatal hernia	0(no)
		1(yes)
X3	Smoking cigarette	0(<1.2 cig./week)
		1(<10 cig./day)
		2(>10 cig./day)
X4	Alcohol consumption	0(<once/month)
		1(once/week)
		2(>once a week)
X5	GER in upright position	Percentage time
X6	GER in supine position	Percentage time
X7	BAO	mEq/h
X8	MAO	mEq/h
X9	PAO	mEq/h
X10	Gastrin	μg/L

c. Multiple regression with stepwise deletion. This analysis was done by means of a statistical package (Statpak 3.1, Northwest Analytical, Inc. Portland, Oregon, U.S.A.). Coefficients of all independent variables were calculated by the multiple regression equation. The variables with very small or negative coefficients were dropped because they were shown to give very weak joint contribution to the dependent variable y. The independent variables with weaker contribution to dependent variable were further deleted from small to large value by backward regression analysis. After above procedures were repeated, the independent variables with weaker contribution to the dependent variable were removed in a step-by-step fashion. In the last step, the remaining independent variables ranked from small to large values.

d. Percentage of contribution. In order to compare the contribution of each independent variable to dependent variable in a concise way, we developed a formula to calculate a percentage contribution of individual independent variable, which was deduced from the equation of multiple regression:

$$y=b0+b1X1+b2X2+\cdots+bkXk$$

When value of the constant factor b0 is assumed as 0, namely, b0 = 0, the multiple regression equation becomes y = b1X1 + b2X2 +⋯+ bkXk, and percentage contribution of individual independent variable to dependent variable y can be calculated by the following formula:

$$\frac{\text{Mean value of individual variable} \times \text{its coefficient}}{\text{Sum of such products for all independent variables}} \times 100\%$$

The larger the percentage value, the more important the corresponding variables in joint contribution.

RESULTS

All patients underwent GI endoscopy, and the endoscopicl results in two groups of patients are listed in Table 2. There was no statistical difference between the two groups of patients as to the endoscopicl findings.

Table 2 Endoscopicl findings in two groups of patients

	Esophagitis		HH(%)	Ulcer (%)
	I-II(%)	III-IV(%)		
E+DU(*n*=31)	74.6	25.4	49.0	100
E(*n*=72)	79.1	20.9	55.6	0
P value	>0.05	>0.05	>0.05	

E+DU = patient with esophagitis and duodenal ulcer
E = patient with esophagitis only

Figure 1 gives the parameters of gastric acid secretion in both groups of patients. There was no significant difference between the two groups of patients in the values of serum gastrin ($P>0.05$). The parameters of 24 h intraesophageal pH monitoring in patients with E+DU and those with esophagitis are shown in Table 3. There was no statistical difference between the two groups as to the parameters of GER ($P>0.05$).

Table 3 Parameters of 24h intraesophageal pH-monitoring

	GER-up* (% time)	GER-sup (% time)	Episode >5 min(No.)
E+DU	15.94±5.91	13.98±14.20	7.85±3.67
E	15.96±14.58	14.33±21.20	8.28±7.10
P value	>0.05	>0.05	>0.05

*GER-up = GER in upright position
GER-sup = GER in supine position

Figure 2 shows the results of multiple regression of dependent variable y (esophagitis) on 10 independent variables. In patients with E+DU, four rounds of regression with stepwise deletion analysis were performed. The first round of regression ended with deletion of independent variables X3 (smoking cigarette), X6 (GER in supine position), X7 (BAO) and X9 (PAO) because they showed very weak joint contribution or negative values. The remaining variables underwent a second round of regression with stepwise deletion, and 2 variables, i.e. X4 (alcohol consumption) and X10 (gastrin) were deleted. In the third round of regression, X8 (MAO) was deleted. The fourth round of regression on the remaining variables was completed, showing the coefficients of variables in order of importance: X2 (HH), 0.5696282; X5 (GER in upright position), 1.027288E-02; and X1 (age), 8.406402E-04. Similarly, in patients with E, the independent variables deleted in the first two rounds of regression were: smoking cigarette, alcohol consumption and gastrin; and age, GER in supine position, BAO and PAO. The third round ended with the following results: X5 (GER in upright position), 1.156702E-02; X8 (MAO), 9.72133E-04; and X2 (HH), 4.629944E-02 (Table 4).

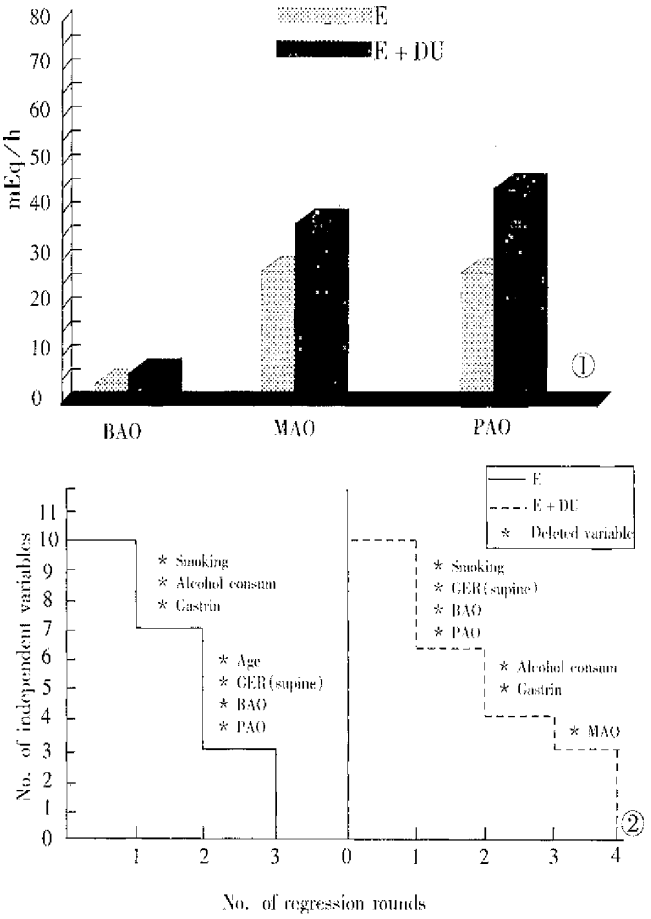

Figure 1 Gastric acid output in two groups of patients.
Figure 2 Flow chart of multiple regression analysis with stepwise deletion.

Table 4 Contribution of independent variable to dependent variable

Variables	Coefficients (b)		*X* value (mean)	Contribution (%)
	1st RR	Last RR		
E+DU				
X2(HH)	0.3943586	0.5696282	0.36363	52.39
X5(GER-up)	4.044132E-02	1.027288E-02	14.65455	38.08
X1(age)	2-869943E-02	8.406402E-04	44.81818	9.53
E				
X5(GER-up)	2.735608E-02	1.156702E-02	19.21875	80.56
X8(MAO)	3.405805E-02	9.72133E-04	28.34812	9.99
X2(HH)	0.1626756	4.629944E-02	0.562600	9.45

RR = round of regression
GER up = GER in upright position

In patients with E + DU, the percentage contributions of independent variables X2, X5 and X1 to y were 52.39%, 38.08% and 9.53%, respectively. Similarly, the percentage contributions of variables X5, X8 and X2 in patients with E to y were 80.56%, 9.99% and 9.54%, respectively.

DISCUSSION

Gastric acid secretion is considered to be an important pathogenetic factor in the development of GER disease. In several studies, a basal acid output higher than normal has been found in patients with reflux esophagitis[17-19]. Collen *et al* have demonstrated that GER patients who did not respond to standard ulce rhealing doses of H2-blocker showed gastric acid hypersecretion[20]. These results stress the point that gastric acid hypersecretion is a crucial factor for GER disease and for the resistance of GER patients to H2-blockers. However, our results showed that although the gastric acid output in patients with E+DU was significantly higher than in patients with E, there was no significant difference between two groups of patients as to severity of esophageal lesions and patterns of GER. These results suggest that no parallel relationship between GAO and severity of GER or esophageal lesions exists in patients with E+DU.

One approach to investigate the reason why the increased gastric acid output is not accompanied by aggravation of both GER patterns and severity of esophageal lesions is to assess quantitatively the role of the various potential pathogenetic factor involved, which may not only influence esophagitis but also interact. Therefore, multiple regression analysis with stepwise deletion is needed. According to this method, the value of any regression coefficient depends on all the other variables included in the regression. With stepwise deletion, the standard partial regression coefficient can be used as a measure of relative importance, the *X* being ranked in order of the size of their coefficients.

In our study, ten independent variables in each patient group were evaluated by multiple regression analysis with stepwise deletion. In patients with E + DU, smoking, GER in supine position, BAO, PAO, alcohol consumption, gastrin, and MAO were deleted in a stepwise fashion because they failed to significantly affect esophagitis. Similarly, in patients with esophagitis without DU, seven variables such as smoking, alcohol consumption, gastrin, etc. were deleted by two rounds of regression. These results indicate that GER in upright position and HH are important determinants of esophagitis. Our results also demonstrate, on the contrary, that the gastric acid output is not an important pathogenetic factor responsible for GER disease.

The relationship between the development of esophagitis and the pattern of GER is also a debatable issue. Some authors have found that the development of esophagitis is related to an increased GER in supine position[21-24]. Others have argued that GER in upright position is the most important pathogenetic factor[25-28]. In our study, the result of multiple regression indicates that the GER in supine position appears to be a weak factor affecting esophagitis, whereas GER in upright position plays an important role in the pathogenesis of esophagitis in both groups of patients.

In conclusion, an increased gastric acid output in patients with E+DU does not aggravate both the pattern of GER and esophageal lesions because the gastric acid output fail to appear as a significant pathogenetic factor responsible for GER disease, whereas GER in upright position and presence of HH are significantly related to GER disease in both groups of patients.

REFERENCES

1 Zaninotto G, DeMeester TR, Schwizer W. The lower esophageal sphincter in health and disease.*Am J Surg*,1988;155(2):104-111
2 Ahtaridis G, Snape WJ, Cohen S. Lower esophageal sphincter pressure as an index of gastroesophageal acid reflux. *Dig Dis Sic*, 1981;26(5):993-998
3 Little AG, DeMeester TR, Kirchner PT. Pathogenesis of esophagitis in patients with gastroesophageal reflux.*Surgery*, 1980;88(1):101-107
4 Berstad A, Weberg R, Fryshov Larsen I. Relationship of hiatus hernia to reflux oesophagitis: a prospective study of coincidence, using endoscopy. *Scand J Gastroenterol*, 1986;21(1):55-58
5 Kaul B, Petersen H, Myrvold HE. Hiatus hernia in gastroesophageal reflux disease.*Scand J Gastroenterol*,1986;21(1):31-34
6 Johnson LF, DeMeester TR, Haggitt RC. Esophageal epithelial response to gastroesophageal reflux, a quantitative study. *Dig Dis*, 1978;23(3):498-509
7 Mittal RK, Lange RC, McCallum RW. Identification and mechanism of delayed esophageal acid clearance in subjects with hiatus hernia. *Gastroenterology*, 1987; 92(1):130-135
8 Sloan S, Kahrilas PJ. Impairment of esophageal emptying with hiatal hernia. *Gastroenterology*,1991;100(5):596-605
9 Howard PJ, Maher L, Prvde A. Symptomatic gastroesophageal reflux, abnormal oesophageal acid exposure, and mucosal acid sensitivity are three separate, though related, aspects of gastro-oesophageal reflux disease. *Gut*, 1991;32(1):128-132
10 Janssens J, Vantrappen G, Ghillebert G. 24-hour recording of esophageal pressure and pH in patients with noncardiac chest pain. *Gastroenterology*, 1986;90(8): 1987-1984
11 Savary M, Miller G. The esophagus. Handbook and atlas of endoscopy. First ed. Switzerland: *Gassmann*, 1987.
12 Bianchi Porro G, Pace F. Comparison of three methods of intraesophageal pH recording in the diagnosis of gastroesophageal reflux. *Scand J Gastroenterol*, 1988;23(5):743-750
13 Pace F, Sangaletti O, Bianchi Porro G. Daytime reduction of gastroesophageal reflux after healing of oesophagitis and its value as an indicator of favourable response to maintenance treatment.*Gut*,1990;31(9):1025-1029
14 Weiser HF, Siewert JR. Investigations with the 24-hour solid-state pH metry: correlation between gastroesophageal reflux extent and reflux sequelae. *Surg Gastroenterol*,1982;1(3):327-334

15 Raufman JP, Collins SM, Pandol SJ. Reliability of symptoms in assessing control of gastric acid secretion in patients with Zollinger-Ellison syndrome. *Gastroenterology*,1983;84(1):108-113
16 Snedecor GW, Cochran WG. Statistical methods. Iowa: The Iowa State University Press, 1979
17 Collen MJ, Ciarleglio CA, Stanczak VJ. Basal acid output in patients with gastroesophageal reflux disease.*Gastroenterology*,1987;92(11):1350(Abstract)
18 Mulholland MW, Reid NJ, Levine DS. Elevated gastric acid secretion in patients with Barrett's mataplastic epithelium.*Dig Dis Sci*,1989;34(12):1329-1335
19 Barlow AP, DeMeester TR, Ball CS. The significance of the gastric secretory state in gastroesophageal reflux disease. *Arch Surg*,1989;124(8):937-940
20 Collen MJ, Lewis JH, Benjamin SB. Gastric acid hypersecretion in refractory gastroesophageal reflux disease.*Gastroenterology*, 1990;98(5):654-661
21 Demeester TR, Johnson LF, Joseph GJ. Patterns of gastroesophageal reflux in health and disease.*Ann Surg*,1976;184(4):459-470
22 Robertson D, Aldersley M, Shepherd H. Patterns of acid reflux in complicated oesophagitis.*Gut*,1987;28(12):1484-1488
23 Lichter I. Measurement of gastro-oesophageal acid reflux: its significance in hiatus hernia.*Br J Surg*,1974;61(3):253-258
24 Pujol A, Grande L, Ros E. Utility of inpatient 24-hour intraesophageal pH monitoring in diagnosis of gastroesophageal reflux.*Dig Dis Sci*, 1988;33(11): 1134-1140
25 De Caestecker JS, Blackwell JN, Pryde A. Daytime gastro-oesophageal reflux is important in oesophagitis.*Gut*,1987;28(5):519-526
26 Branicki FJ, Evans DF, Jones JA. A frequency-duration index (FDI) for the evaluation of ambulatory recordings of gastro-oesophageal reflux. *Br J Surg*,1984;71 (4):425-430
27 Rokkas T, Anggiansah A, Uzoechina E. The role of shorter than 24-h pH monitoring periods in the diagnosis of gastro-oesophageal reflux. *Scand J Gastroenterol*, 1986;21(6):614-620
28 Blackwell JN, Heading RC. When does gastro-oesophageal reflux occur in patients with peptic oesophagitis? (Abstract). *Gut*, 1980;21(5):A922

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Classification of gastric neuroendocrine tumors and its clinicopathologic significance

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Subject headings stomach neoplasms/ pathology; neuroendocrine tumors/pathology; carcinoid tumor/pathology; gastritis, atrophic; G cell; immunohistochemistry; microsocopy, electron

Abstract

AIMS To study the pathologic classification of gastric neuroendocrine tumors and its clinicopathologic significance.

METHODS Paraffin-embedded sections of 52 gastric neuroendocrine tumors including 42 carcinoid tumors, and 10 cases of neuroendocrine carcinoma from 326 patients who underwent resection of stomach carcinomas were studied by immunohistochemical methods including 10 endocrine markers or hormones antibodies and endocrine cells in gastric neuroendocrine tumors and extratumoral mucosa were observed under electromicroscope.

RESULTS The 52 gastric neuroendocrine tumors were divided into three types: ① Gastrin dependent type of carcinoid (26 cases) accompanied by chronic atrophic gastritis (CAG) and tumor extension limited to the mucosa or submucosa complicated with hypergastrinemia and G cell hyperplasia. This type was consistently preceded by and associated with generalized proliferation of endocrine cells in the extratomoral fundic mucosa. ② Non-gastrin dependent type of carcinoids (16 cases) associated with neither CAG nor hypergastrinemia. This type was more aggressive; and ③ Neuroendocrie carcinomas (10 cases), which are highly aggressive tumors.

CONCLUSIONS A correct identification of different types of gastric endocrine tumors has major implications for the treatment and prognosis of the patients.

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INTRODUCTION

According to recent literature, gastric carcinoids incidence ranges from 11% to 41% of all gastrointestinal carcinoids[1,2]. Until a decade ago, however, it was regarded as pathological curiostry accounting for about 3%[3]. It was recognized that gastric neuroendocrine tumors cover a spectrum of neoplasms showing wide variations in their clinicopathological features, prognosis and pathogenetic mechanisms[4,5]. The classification of gastric neuroendocrine tumors (GNET) is of some value in its treatment and prognosis[6]. This paper is to study clinicopathologic classification of GNET for the correct management of patients.

MATERIALS AND METHODS

A total of 42 cases of gastric carcinoid were collected in Parma University, Italy (29 cases) and China (13 cases). Ten cases of neuroendocrine carcinoma were identified from 326 resected gastric carcinomas by immunostaining and electromicroscopy.

Formalin-fixed and paraffin-embedded tissues were available and the blocks of specimens included tumor and extratumoral mucosa. A series of 15 sections (5μm thick) were cut from each block. Hematoxylin and eosin, Acilan blue pH 2.5-periodic staining were carried out. The cellular localizations of neuroendocrine substances were detected by the avidinbiotin^{a2}peroxidase complex method (ABC kit, K355; Daco, Carpenteria, CA, USA). The primary antibodies and positive controls are listed in Table 1. All antibodies were polyclonal except serotonin and bombesin. Peroxidase was revealed using diaminobenzidine tetrahydrochloride (DAB) and H202. Control of specificity of the immunoreaction was performed by incubating consecutive sections with nonimmune serum instead of the primary antiserum or with the specific antisera preabsorbed with the excessive amounts of the respective antigens.

Eight cases of the 10 neuroendocrine carcinomas were examined under transmission electromicroscopy (Musashino, Tokyo, Japan).

Fasting serum gastrin was determined in 33 of 42 carcinoid patients and 8 of 10 neuroendocrine carcinomas.

RESULTS

A total of 52 GNET cases were divided into three types according to Bordi[4] and Rindi[5]: Type 1, gastrin dependent carcinoid; type 2,non-gastrin de-

pendent carcinoid; and type 3, neuroendocrine carcinoma. The immunostaining results for neuroendocrine markers and hormone antibodies are summarized in Table 2. Diffuse features of neuroendocrine differentiation were shown in all the 52 cases and ultrastructural evidence of endocrine type of cytoplasmic granules was observed in 8 cases of neuroendocrine carcinomas examined with transmission electronmicroscopy. Those tumors were classified as neuroendocrine tumors in stomach.

Table 1 Antibodies used in the present study

Code	Antigens	Working dilution	Positive control
M869	CgA	1:400	Pancreas
	SP	1:400	Pancreas
A568	GAST	1:300	Gastric antrum
A566	SS	1:400	Pancreas
A756	5-HT	1:20	Small bowel
A576	CT	1:800	Medullary carcinoma of thyroid gland
A619	PP	1:4000	Pancreas
A231	β-HCG	1:800	Human placenta
A571	ACTH	1:800	Pituitary gland
CA-08-219	BOM	1:20000	Duodenum

CgA: chromogranin A; SP: synaptophysin; GAST: gastrin; SS: somatostatin; 5-HT: serotonin; CT: calcitonin; PP: pancreatic polypeptide; β-HCG: human chorionic gonadotroph; ACHT: adrenocorticotrophin; GLU: glucagon; BOM: bombesin

Table 2 Immunohistochemical results in 52 cases of gastric tumors

	Type 1 (26)				Type 2 (16)				Type 3 (10)			
	+	±	-	ND	+	±	-	ND	+	±	-	ND
CgA	24	1	0	1	14	1	0	1	9	1	0	0
SP	16	3	4	3	4	6	4	1	2	5	0	3
GAST	4	2	17	3	2	1	11	2	1	1	8	0
SS	3	0	22	2	1	0	14	3	1	1	8	0
5-HT	10	1	15	0	9	0	5	2	2	0	7	1
CT	1	0	11	14	0	1	4	11	1	1	8	0
β-HCG	5	1	19	1	2	1	12	1	4	2	4	0
ACTH	1	1	12	12	0	1	3	12	2	1	5	2
PP	4	0	21	1	2	1	5	4	1	1	5	3
GLU	0	0	25	1	0	0	15	1	1	0	9	1
BOM	1	1	8	16	0	1	6	9	1	1	7	1

+: positive; -:negative; ±: doubtful; ND: study not done

Type 1. Gastrin dependent carcinoid (26 cases), with a mean age of 58.2 years (range 27-88 years), a female to male ratio of 1.36:1. Eleven cases are single tumors appearing as polypoid lesions. Eight of them were <1cm in maximum diameter, 2 were 1 to 1.5cm, and only one was 2.5 cm. Fifteen multiple tumors were 1.0cm - 1.5cm in each one. In histological structure the carcinoids appeared either trabecular or in solid arrangement (Figure 1). Tumor cells were monomorphic and medium sized in regular shape and with round nuclei. Mitoses were either absent or seen occasionaly. Sixteen cases confined to the gastric mucosa and 4 cases to the submucosa. One case was found to have lymph node metastases, and no distal metastases was found.

This type of carcinoids is associated with CAG and proliferation of endocrine cells in extratumoral fundic mucosa. It includes hyperplasia as simple, linear, micronodular and adenomatoid hyperplasia and dysplasia as enlarging, fusing micronodules, invasive lesion and nodule with newly formed stroma. When at a diameter of 0.5 mm, it was defined as carcinoid tumor. The hyperplasia and dysplasia (Figure 2) of endocrine cells in extratumoral mucosa were found in all 26 cases. The fasting serum gastrin of 21 of 26 cases were examined before operation and all had hypergastrinemia, ranging from 700ng/L-1000ng/L (normal is 100ng/L). G cell hyperplasia in antrum was observed in 24 cases and antrum mucosa was investigated. Nine of 11 cases of single tumors underwent polypectomy, and 2 cases partial gastrectomy, and 15 multiple tumors received antrectomy. Eighteen cases were followed up, there were 3 deaths with a mean survival of 40 months, which was unrelated to the gastric carcinoid. After a mean of 64 months, 15 were alive.

Type 2. Non-gastrin dependent carcinoid (16 cases). The mean age was 53.8 years (range 42-61 years), a female to male ratio of 0.6:1. All tumors were single with a median diameter of 2.5cm. Tumors showing moderate enlargement of lobules and trabeculae and moderate cellular atypia included nuclear polymorphism, hyperchromasia, prominent nuceoli and slight increase in mitotic count (<1 per 2 HPF) (Figure 3). Small necrosis may also be found. The muscularis propria or serosa were invaded in 11 of 16 cases (68.8%). Local lymph node metastases were found in 9 (56%) cases and distant metastases were seen in 5 (31%) patients. The circulating gastrin of 12 of 16 patients was examined and had normal levels. No antral G cell hyperplasia was found in 13 of 16 cases. Two of 16 patients displayed hyperplasia of endocrine cells in extratumoral mucosa and none had dysplasia. Among 14 cases followed up died with a mean survival of 32 months and 10 were alive after a mean follow-up of 72 months.

Type 3. Neuroendocrine carcinomas (10 cases). The mean age was 62.8 years (range 57-78 years), 8 men and 2 women. All tumors were single, with an average size of 5.1cm (range 3.0cm - 8cm). Histologically, they were characterized by large, poorly defined, solid aggregates or diffuse shets of round or spindle cells (Figure 4). Mitoses were usually abundant (>1 per HPF). Focal necrosis was common. The extratumoral mucosa was associated with chronic atrophic gastritis in six cases. Ultrastructural evidence of endocrine type cytoplasmic granules was found in 8 cases examined. The hypergastrinemia was found in only one of 5 cases.

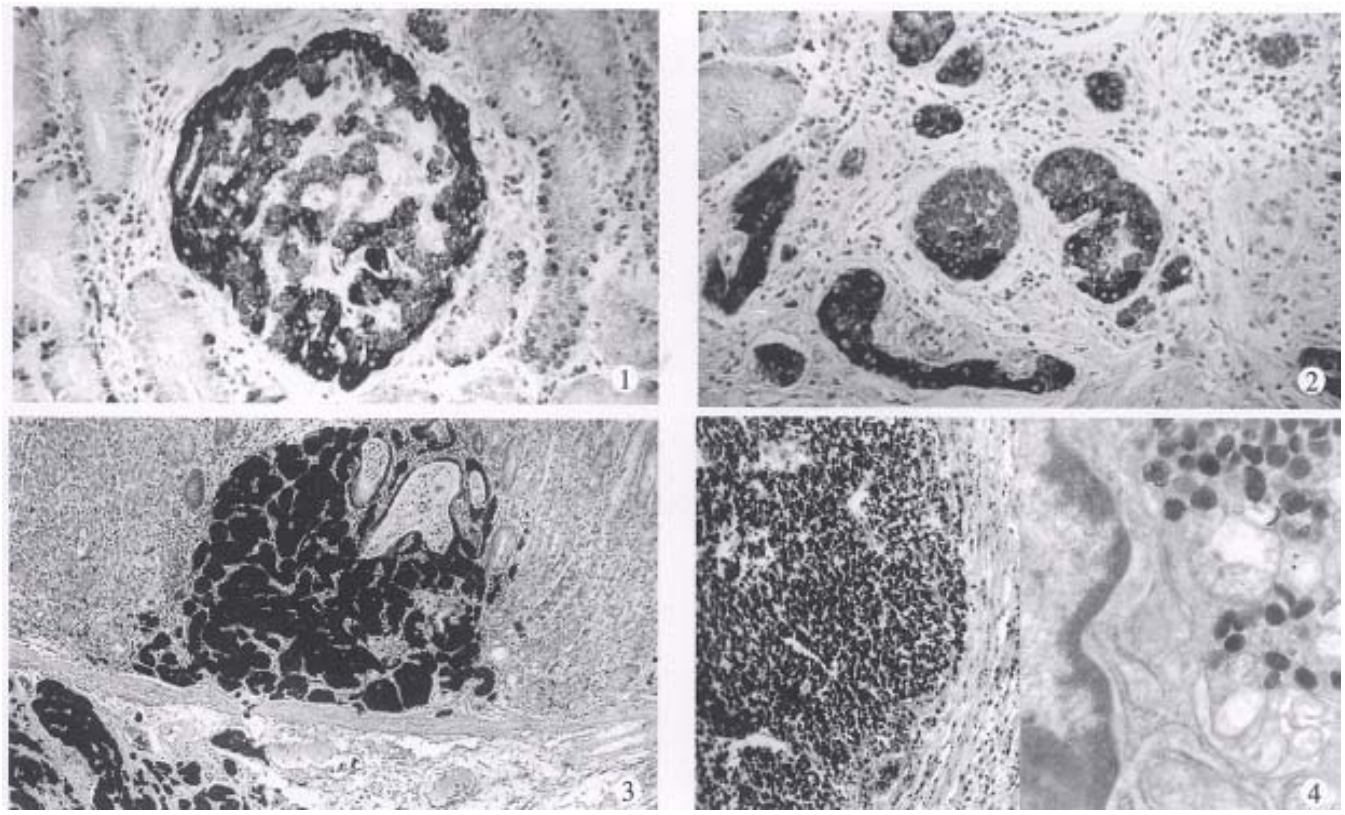

Figure 1 Type 1 gastric neuroendocrine tumorsgastrin dependent carcinoid. Histology shows typical carcinoid with trabecular arrangement. Tumor confined to the gastric mucosa, many tumor cells had positive reaction for chromogranin A. ABC method ×450

Figure 2 The hyperplasia and dysplasia of endocrine cells in extratumoral mucosa were found in type 1 gastrin dependent carcinoid. Endocrine cells show positive reaction for chromogranin A. ABC method ×450

Figure 3 Type 2 non-gastrin dependent carcinoid. Tomor invaded the muscularis propria. The hyperplasia of endocrine cells are not distinct. Diffuse synaptophysin in immunostaining of tumors. ABC method ×250

Figure 4 Type 3 neuroendocrine carcinoma. Tumor cells are small and round, mitoses are abundant. Focal necrosis was found. HE×250 (left). Ultrastructural appearance of the neuroendocrine carcinoma with characteristic secrectory granules. ×28000 (right)

Table 3 Clinicopathological characters of gastric neuroendocrine tumors

Type	Sex (F:M)	Mean age (a)	Tumor number	Infiltration	Metastases	Death	Treatment
1	1:3.6	58.2	Single	m,sm		no	Polypectomy
			Multiple	m,sm	3.8%	no	Antrectomy
1	0.6:1	53.8	Single	mp,s	56%	28%	Gastrectomy
3	1:4	62.8	Single	mp,s	80%	70%	Gastrectomy Chemotherapy

m: mucosa; sm: submucosa; mp: muscularis propria; s: sierosa

Carcinomas invaded the gastric wall, reaching the muscularis propria in three cases (30%), and serosa in seven (70%). Eight cases showed metastases to local lymph nodes and two to livers. Deaths were observed in seven cases with a mean survival of 8 months (range 2-10 months) after a mean follow-up of 54 months.

Table 3 and Figure 5 summarize the clinicopathological characters and survival of variations of gastric neuroendocrine tumors.

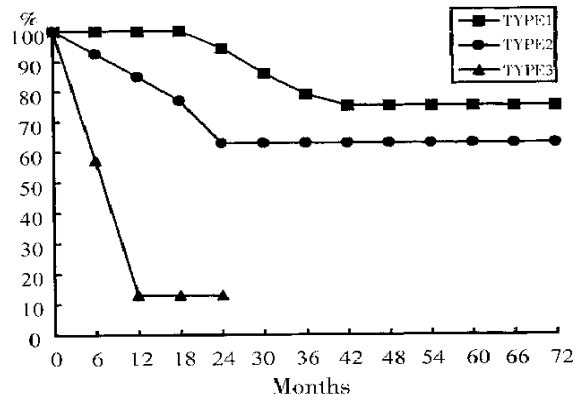

Figure 5 The survival curve of three types of gastric endocrine tumors.

DISCUSSION

The classification of gastric neuroendocrine tumors is subdivided into carcinoid and neuroendocrine carcinoma. Two subtypes of carcinoid tumors including gastrin dependent carcinoid and non-gastrin dependent carcinoid has further recognized in the study. Rindi *et al*[1] reported that carcinoids were subdivided into three subtypes which included: carcinoid tumors associated with atrophic gastritis[2]; carcinoid tumors associated with Zollinger Ellison syndrome[3,4]; carcinoid tumors of sporadic type. The carcinoid with ZES appears absent in ours.

The result of features of 26 cases of gastrin dependent carcinoid type is in agreement with some previous reports in which it was named as carcinoids associated with atrophic gastritis. This type is either single (polypoid lesions) or multiple tumor. The histological features show typical carcinoids. Tumor extension limited to the mucosa or submucosa. Gastrin dependent carcinoids are consistently associated with generalized proliferation of endocrine cells in the extratumoral fundic mocosa. A histopathological classification has been formulated for the spectrum of proliferative lesions presented by fundic endocrine cells of hypergastrinemic patients. The classification arranged in a sequence presumed to reflect the temporal evolution of the process and the increasing oncologic risk for the patients[5,6]. Our data also provide a rationale for the treatment of single carcinoids with tumorpolypectomy followed by regular endoscopic follow-up. In the patients with multiple tumors, antrectomy is recommended. Postantrectomy regression of proliferating gastric endocrine cells was documented in several cases.

The 16 cases of non-gastrin dependent type which correspond to Solcia's carcinoids of sporadic type[7,8] had single tumors. They usually presented with typical carcinoid features by slight increase in mitotic count (<1 per HPF). Serum levels of gastrin were in the normal range. These tumors appear free from hypergastrinemia and should not benefit from antrectomy. Their aggressive behavior dictated a complete surgical ablation.

The type of neuroendocrine carcinomas composed mainly of cells was strongly positive for endocrine markers (Cg A, SP or hormonal products). Those positive cells were major parts of the tumor (>50%). Although neuroendocrine differentiation is not a rare phenomenon in common gastric carcinoma (3.1%-53.5%)[9] but neuroendocrine carcinoma of the stomach was found infrequently. In this series, 10 (3.1%) were identified from 326 gastric carcinomas. The endocrine granules were observed by trasmission electron microscopy. This type of carcinomas is high-grade one. Tumors were considered aggressive cancers with poor prognosis showing large tumors (mean 5.1cm) with deep invasion and metastasis. The surgical resection is the most appropriate form of treatment for this type and the usefulness of multiagent chemotherapy remains to be evaluated in larger series of patients.

Immunohistochemically, no significant specific differences were found in the three types of neuroendocrine tumors of stomach[4,10]. Types 1 and 2 of carcinoid were mostly composed of ECL cells which produce histamine[5,6]. The eight hormonal antibodies used in the study showed a various amount of positive expression in neuroendocrine tumors of stomach. Further investigations are needed to elucidate the significance of hormones produced by those tumors.

In conclusion, neuroendocrine tumors of the stomach cover a spectrum showing wide variations in their clinicopathological features, prognosis, and pathogenetic mechanisms. It has implications for the treatment.

REFERENCES

1 Rindi G, Luinetti O, Cornaggia M, Capella C, Solcia E. Three subtypes of gastric argyriphil carcinoid and the gastric neuroendocrine carcinoma: clinicopathologic study.*Gastroenterology*,1993;104(4):994-1006

2 Solcia E, Fiocca R, Sessa F, Rindi G, Gianatti A, Cornagia M et al. Morphology and natural history of gastric endocrine tumors. In: Hakanson R, Sundler F, eds. The stomach as an endocrine organ. *Amsterdam: Elsevier,* 1991:473-498

3 Godwin JD. Carcinoid tumors: an analysis of 2837 cases. *Cancer,* 1975;49(2): 560-569

4 Bordi C, Yu JY, Baggi MT, Davoli C, Pilato FP, Baruzzi G. Gastric carcinoids and their precursor lesions. A histologic and immunohistochemical study of 23 cases.*Cancer*,1991;67(3):663-672

5 Rindi G, Bordi C, Rappel S, Rosa SL, Stolte M, Solcia E. Gastric carcinoids and neuroendocrine carcinomas: pathogenesis, pathology, and behavior. *World J Surg*,1996;20(2):169-172

6 Bordi C. Endocrine tumours of the stomach.*Pathol Res Pract*,1995;191(2): 373-380

7 Creutzfeldt W. The achlorhydria carcinoid sequence: role of gastrin. *Degestion*, 1988;39(1):61-79

8 Solcia E, Rindi G, Fiocca R, Villani L, Buffa R, Ambrosiani L et al. Distinct patterns of chronic gastritis associated with carcinoid, neuroendocrine carcinoma or ordinary cancer and their role in tumorigenesis. *Yale J Biol Med*,1992; 112(3):793-1047

9 Luping W, Jiyao Y, Jingquan S, Yanie L. Neuroendocrine cells in gastric carcinoma and in extratumoral gastric mucosa.*Cell Visson*,1996;3(1):47-53

10 Staren ED, Lott S, Saavedra VM, Jansson DS, Deziel DJ, Saclarides TJ *et al*. Neuroendocrine carcinomas of the stomach'aclinicopathologic evaluation. *Surgery*,1992;112(6):1039-1047

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TRH microinjection into DVC enhances motility of rabbits gallbladder via vagus nerve *

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Subject headings gallbladder; vagus nerve; dorsal vagal complex; thyrotropin releasing hormone; cholinergic M receptors; rabbits

Abstract

AIM To investigate the effects of TRH in DVC on motility of the gallbladder in rabbits.

METHODS fter fasted for 15h-18h, rabbits were anesthetized with urethane (1.0g/kg). Gallbladder pressure (GP) was measured by a frog bladder perfused with normal saline.

RESULTS After microinjection of TRH (8.8nmol, 1μl) into DVC, GP was raised and the frequency of phasic contraction of gallbladder (FPCGB) increased. All the doses of TRH (0.13, 0.25, 0.50, 0.80, 1.30nmol, 1μl) injected into DVC could excite the motility of gallblader. As the dose of TRH was enlarged, the amplitude and duration of the reaction increased. Effects of TRH in DVC on motility of the gallbladder could be completely abolished by atropine (0.2mg/g, i.v.) or vagotomy, but could not be inhibited by phentolamine iv (1.5mg/g) or propranolol iv (1.5mg/g)or by transecting the spinal cord.

CONCLUSION Thyrotropin-releasing hormone in DVC can excite motility of gallbladder. This effect was mediated by vagus nerves and peripheral M receptor. Its physiological significance may be related to maintaining the phasic contraction of gallbladder in interdigestive period.

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INTRODUCTION

Our previous studies indicated that the dorsal vagal complex (DVC) might play an important role in regulating motility of extrahepatic biliary tree[1], but we do not know which neurotransmitter mediates this effect.

Thyrotropin-releasing hormone (TRH) is one of important brain gut peptides[2]. There is a high density of TRH-immunoreactive nerve terminals in DVC[3]. Injecting TRH into DVC could excite the motility and secretion of stomach[2]. However, there is no report about TRH in DVC on the motility of extrahepatic biliary tree. In this study, TRH was injected into DVC of anesthetized rabbits, and then the effect on mean gallbladder pressure (GP) and frequency of phasic contraction of gallbladder (FPCGB) were observed, and the peripheral route of the effect of TRH was also investigated.

MATERIALS AND METHODS

Animal preparation

The experiments were conducted in 38 rabbits weighing 2.0 kg - 2.5 kg. Fasted for 15 h - 18 h, but allowed to drink water, the rabbits were anesthetized with urethane (1.0g/k, iv.). A cannula was inserted into the trachea. Unilateral femoral artery was catheterized for measurement of blood pressure. The rectal temperature was maintained at 37℃-38.5℃.

Measurement of GP

A frog bladder filled with saline was placed in gallbladder and connected to a transducer (TP-200T) by a catheter. The blood pressure and GP were recorded simultaneously by a polygraph (RM-6000, NIHON KOHDEN).

Microinjection of TRH

The animal's head was fixed in a stereotaxis (I-C model, JIANG-WANG, China). According to the Messen's methods, a stainless steel guide tube (0.5mm in outer diameter) containing a needle was inserted into DVC (rostral from obex 0.5mm-1.0mm, left or right from midline 0.7mm-1.2mm, depth from the surface 0.5mm-1.2mm). Normal saline or TRH solution(1μl) was injected into DVC by a microsyringe connected to a cannula (0.3mm in outer diameter).

Statistical analysis

Before the following tests were made, the basal level of GP of each animal was taken as control (i.e., basal GP as 0 kPa in place of real level). GP increase during the test, was considered as positive (+) value, while GP decrease was taken as negative (-). Student's *t* test was used for the statistical analysis. All values were $\bar{x}\pm s_{\bar{x}}$. $P<0.05$ was considered to be statistically significant.

RESULTS

Normal GP

In interdigestive period, two kinds of motion of gallbladder were found, the tonic and phasic contractions. Tonic contraction, which means continuous and weak contraction, could maintain GP at a stable level ($2.67\times10^3\pm190$Pa, $n = 8$). Phasic contraction, with a duration of 5-20s and frequency of 1-3 times per minute, raised GP by 60kPa - 200kPa rhythmically (Figure 1a).

Microinjection of TRH into DVC

After TRH (0.8nmol,1μl) was microinjected into DVC, the motility of gallbladder was enhanced. The effect occurred in 1min after the injection, reached its peak in 10 minutes and returned to normal within 20min-30min. At 5min after injection, GP increased by 167kPa ± 29kPa ($n = 5$, $P < 0.02$); FPCGB increased from 1.14±0.26 to 2.28 ± 0.26 per min ($n = 5$, $P<0.034$) (Figures 1b, 2-3). However, after normal saline (1μl) injected into DVC or TRH (0.8nmol, 1μl) into the control position in medulla (lateral 0.5mm-1.0mm to the DVC), the GP and FPCGB did not change significantly.

Dose-response of TRH microinjected into DVC on the GP and FPCGB

All the doses of TRH (0.13, 0.25, 0.50, 0.80, 1.30nmol, 1μl) microinjected into DVC could excite the motility of gallbladder. As the dose increased, the strength and duration of the effect of TRH on GP and PCGB went up, with a significant dose-response (Figures 4 and 5).

Peripheral route of the TRH effect on the motility of gallbladder.

Ten minutes after the cervical vagotomy ($n = 5$) or i.v. injection of atropine (0.2g/kg, $n = 5$), microinjection of TRH (0.8nmol, 1μl) could no longer excite the motility of gallbladder. Within 30 minutes after the injection of TRH, the GP and FPCGB had no significant changes (Figure 1b, c). However, ten minutes after transection of the spinal cord from T1 or i.v. injection of phentolamine (1.5 mg/kg) or propranolol (1.5 mg/kg), injection of TRH into DVC could also enhance the motility of gallbladder (Figure 1d, e, f).

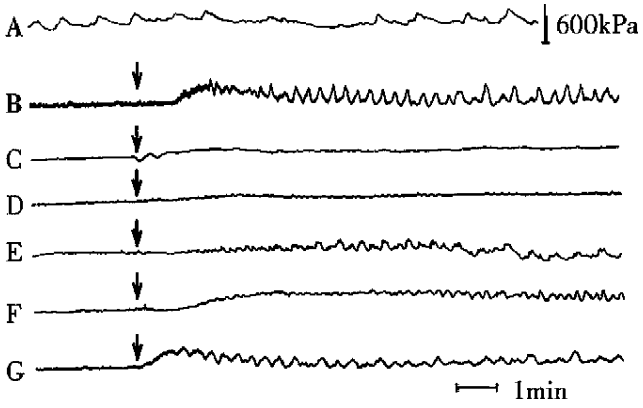

Figure 1 The effects of TRH microinjected into DVC on the GP. A. control; B. TRH (0.8 nmol/1 μl); C. i.v. atropine (0.2 mg/kg); D. vagotomy; E. i.v. phentolamine (1.5 mg/kg) ; F. i.v. propranolol (1.5 mg/kg); G. after transection of spinal cord through T1; G. ↓ (0.8 nmol/1 μl).

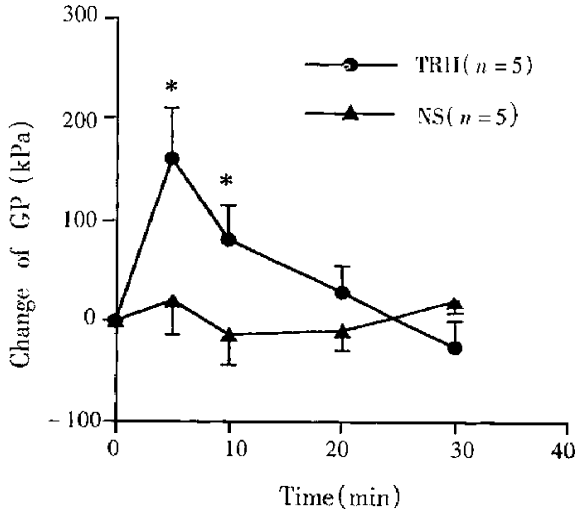

Figure 2 The effects of microinjection of TRH (0 .8 nmol/1 μl) or NS on GP. *$P<0.05$ as compared with NS

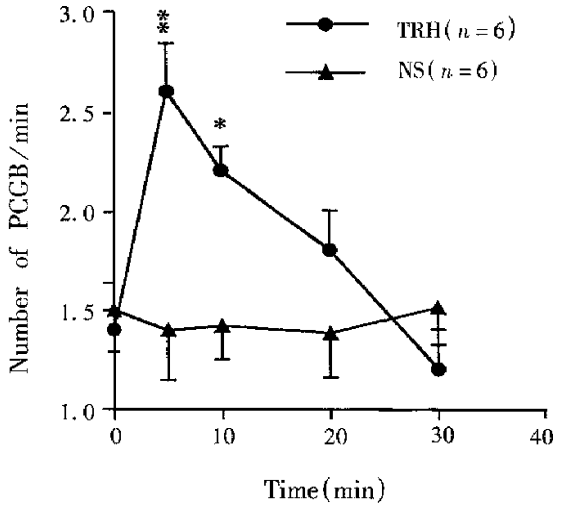

Figure 3 The effect of microinjection of TRH (0.8 nmol/1 μ1) or NS on the frequency of phasic contraction of gallbladder (FPCGB). **$P<0.01$, *$P<0.05$ as compared with NS

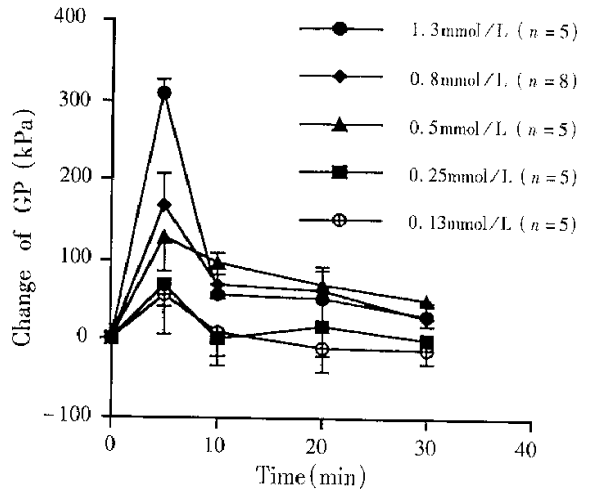

Figure 4 Dose-response curve for TRH stimulation on GP after microinjection into DVC.

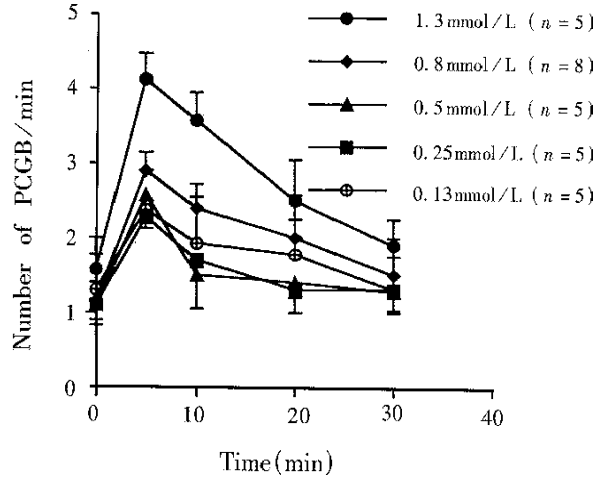

Figure 5 Dose-response curve for TRH stimulation on the number of phasic contraction of GB (PCGB) after microinjection into DVC.

DISCUSSION

In this study, we found that TRH microinjection into DVC raised GP and FPCGB. As an important brain-gut peptide, TRH in DVC plays an important role in regulating the function of gastrointestinal tract[2]. We found for the first time that TRH microinjected into DVC excited the motility of gallbladder in rabbits.

The effects of TRH microinjected into DVC on the motility and secretion of gut may be physiological. In DVC, there is a high density of TRH-immunoreactive nerve terminals which come from the nucleus raphe pallidus and raphe obscurus. Some of these terminals make synaptic contacts on the dendrities of gastric vagal motoneurons[5]. Multicolon TRH antibody injected into DVC can inhibit the gastric acid secretion[6].

Through vagus nerve and peripheral cholinergic M receptors, TRH could excite the gallbladder motility, but its physiological significance is unknown. Bile evacuation occurrs during sham feeding, and is abolished by vagotomy[7]. After vagotomy or gastrectomy, the incidence of gallstones increases[8,9]. In this experiment, TRH in DVC raised GP and then facilitated the bile evacuation. Thus TRH may be a possible candidate for the transmitter in DVC mediating the cephalic bile output.

The present experiment showed that there was rhythmic phasic contraction of gallbladder in rabbits in the interdigestive period, which was similar to that found in dogs[10]. It was believed that this phasic contraction stirred the bile in gallbladder and prevented gallstones forming in the interdigestive period[11]. Our experimental results also showed that TRH microinjected into DVC enhanced the phasic contraction via vagus nerve. Thus TRH in DVC may be regarded as an important neurotransmitter in mediating the phasic contraction of gallbladder. Similar to Ura's data in dogs[11], vagotomy almost inhibited the phasic contraction completely in our study. It was suggested that the tonic excitation of vagus nerve center may be important in maintaining this type of motion in the interdigestive period. TRH may participate in this effect.

In conclusion, we believed that TRH in DVC may play an important role in regulating the motility of gallbladder via vagal nerves and peripheral cholinergic M receptors, and may participate in cephalic gallbladder emptying during ingestion and maintain the phasic contraction of gallbladder in the interdigestive period.

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REFERENCES

1 Liu CY, Liu JZ, Zhou JH, Wang HR, Li ZY. The effects of electric and chemical stimulation of DVC on the motility of biliary system. *Acta Acad Med Shandong*, 1997;35(3):207-210
2 Tache Y, Robertm L, Stephens TR, Ishikawa T. Central nervous system action of TRH to influence gastrointestional function and ulceration. *Ann NY Scand Sci*,1989;553(2):269-295
3 Richard BL, Margaret SK, Richard RM. Thyrotropin-releasing hormone-immunoreactive projections to the dorsal vagal motor nucleus and the nucleus of the solitary tract of the rat. *J Comp Neuro*,1991;311(2):271-288
4 Palkovits M, Mezey E, Eskay R, Brwnstein MJ. Innervation of the nucleus of the solitary tract and the dorsal vagal nucleus by thyrotropin-releasing hormone-containing raphe neurons. *Brain Res*,1996;373(2):246-251
5 Rinaman L, Richard RM. TRH immunoreactive nerve terminals synaps on the dentrities of the gastric vagal motoneurons in the rat. *J Comp Neuro*, 1990;294 (2):235-251
6 Hernasez DE, Jenjers L, Emerick SG. Inhibition of gastric acid secretion by immunoneutralization of endogenous brain TRH. *Brain Res*, 1987;401(I3):381-384
7 Fisher RS, Rock E, Malmud LS. Gallbladder empting response to sham feeding in humans. *Gastroenterology*,1986;90(5):1854-1857[a¤]
8 Hauters P, Roden A, Pourbaix A, Aupaix F, Couman P. Therasse G cholelithiasis: a serious complication after total gastrectomy. *Br J Surg*, 1988; 107(3):899-900
9 Ihasz M, Griffith CA. Gallstones after vagotomy. *Am J Surg*, 1981;141(1): 48-50
10 Matsumato T, Sarna SK, Condon RE, Dodds WJ, Mochinaga N. Caine gallbladder cyclic motor activity. *Am J Physiol*,1985;255(2):G409-416
11 Ura K, Sarna K, Condon RE. Antral control of gallbladder cyclic motor activity on the fasting state. *Gastroenterology*, 1992;102(1):295-302

Cloning and expression of NS3 cDNA fragment of HCV genome of Hebei isolate in *E. coli*

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Subject headings hepatitis C virus; NS3 gene; gene expression; DNA, viral; viral proteins; sequence analysis; polymerase chain reaction; enzyme-linked immunosorbent assay; *Escherichia coli*

Abstract

AIM To obtain greater antigenicity of HCV NS3 protein.

METHODS The HCV NS3 cDNA fragment was amplified by reverse transcription polymerase chain reaction from the sera of the HCV infected patients. The DNA sequence was determined by dideoxy-mediated chain termination method using T7 polymerase. HCV NS3 protein was expressed in *E. coli*.

RESULTS Sequence analysis indicated that the HCV isolate of this study belongs to HCV-II; SDS-PAGE demonstrated an Mr 23 800 and an Mr 22 000 recombinant protein band which amount to 14% and 11% of the total bacterial proteins separately. Western blotting and ELISA showed NS3 protein possessed greater antigenicity.

CONCLUSION Recombinant HCV NS3 protein was expressed successfully, which provided the basis for developing HCV diagnostic reagents.

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INTRODUCTION

Since HCV (hepatitis C virus) was identified as the major cause of post-trans fusional non-A, non-B hepatitis by Choo *et al* in 1989[1], the harm of this infectious disease to the human health has been gradually realized. Up to 50%-60% of the HCV hepatitis cases turn chronic, developed into cirrhosis and hepatocellular carcinoma. Due to lack of effective therapeutic methods, it is exceptionally important to prevent the transmission of HCV. As HCV is mainly transmitted through blood sources, the key to preventing the transmission of HCV is to screen the blood donors using specific and sensitive HCV diagnostic reagents. HCV NS3 antigen is necessary in anti-HCV diagnostic reagents because of the lower divergence in NS3 region of HCV genome and the strong antigenicity of NS3 protein. In addition, because of the early appearance and high incidence of the antibody against NS3 antigen. In this report, the HCV NS3 gene fragment was cloned and expressed in *E. coli*. The obtained NS3 antigen showed strong antigenicity, which provided the basis for developing HCV ELISA reagents.

MATERIALS AND METHODS

Sera and extraction of HCV RNA

Serum for cDNA synthesis was derived from a patient who comes from Hebei Province, which is positive for anti-HCV and HCV RNA. HCV RNA was extracted by single step of guanidinium-phenol-chloroform method[2]. Ninety-four serum specimens for ELISA were screened from 460 sera derived from patients with different types of hepatitis, which are anti-HCV positive tested by Abbott second generation HCV ELISA, provided by the Institute of Hepatitis, Youan Hospital.

Expression vectors

Expression vectors are pRX vectors with trpE promotor[3] and pMY vector with p_Rp_L promotor. p_{RX} vector expresses fusion protein with 18 amino acid at N-termini of the protein induced by IPTG. pMY vector expresses non-fusion protein induced by heat at 42℃.

Primers designed for reverse transcription polymerase chain reaction

HCV NS3 protein antigenicity analysis was conducted with computer according to HCV-BK[4] sequence.

Refered to the analysis results, the conservative region was selected and the primers were designed as follows:

Outer primer:F1 5'GTTGCGAAGGCGGTGGACTT 3'
R1 5'GTCGTCTCAATGGTGAAGGT 3'
Inner primer:F2 5'GGAATTCTCCGGCTGCATATGCA 3'
R2 5'CCATCGATAGGTATAGCCCGTCAT 3'

To facilitate subcloning, EcoR I and Cla I restriction endonuclease sites were added at the 5'-termini of the inner primer. The outer reverse primer was used in both cDNA synthesis and cDNA amplification.

Reverse transcription polymerase chain reaction

According to the methods by Widell and Cristiano[5,6], before reverse transcription, RNA template was denatured at 94℃ for 5 min, then on ice promptly. Reverse transcription and first round of PCR were run in the same buffer at the same tube. Reaction volume was 100μl, containing AMV 1.6U and Taq DNA polymerase 2.5U. The reaction order is reverse transcription at 42℃ for 30min, inactivation of reverse transcriptase 94℃ for 3min, and PCR consisting of 94℃ 55s, 42℃ 1min, 72℃ 1.5min for 5 cycles, and 94℃ 55s, 55℃ 1min, 72℃ 1.5min for another 30 cycles. One tenth of the first round of PCR products were used at template to conduct the second round of PCR, the conditions were the same as the last 30 cycles of the first round of PCR. Finally, PCR products were analyzed on 15g/L agarose gel electrophoresis.

Sequencing of cDNA fragment

The cDNA fragment was subcloned into M13 mp 18/19 vectors and single stranded DNA template was prepared. Sequencing reaction was performed using T7 sequencing kit. Nucleotide and amino acid homology analysis were conducted using Goldkey program.

Construction of recombinant plasmids and expression of proteins

cDNA fragments and vectors were cleavaged by restrictive endonuclease, ligated with T4 DNA ligase, the *E. coli* HB101 was transformed. The recombinant plasmids were identified through restrictive endonuclease digestion[7]. *E. coli* HB101 carrying recombinant plasmid were inoculated in M9 media. After incubation at 30℃ for about two hours, the 3β-indolyl acrylic acid was added to the final concentration of 10mg/L, and the bacteria were cultured for 5 hours to express the protein. E. *coli* HB101 carrying recombinant plasmid pMYNS3 were cultured at 30℃ for about two hours, then transferred to 42℃ for another 5 hours to express the recombinant protein.

Identification of the expressed proteins

Twelve percent SDS-PAGE and Western blotting were used to identify the expressed proteins. The sample was prepared as follows: removing 1ml of induced culture, centrifuging to collect the pellet, resuspending the pellet and boiling for 5min to lyse the bacteria with 2×SDS loading buffer. After the electrophoresis, the polyacrylamide gel was split into two pieces. One piece of gel was stained with Coomassie Brilliant Blue R-250, and scanned to analyze the expression level of the recombinant protein. The other piece was blotted to nitrocellulose membrane. Blotting condition was $0.8m \cdot cm^2 \cdot 1.5$ hours. The blotted membrane was incubated with blocking solution containing 10ml/L fatal bovine serum for one hour, then incubated with anti-HCV positive serum (diluted 1: 100) at 4℃ overnight. The following day, the membrane was incubated with sheep anti-human IgG-HRP (diluted 1:500) at room temperature for 30min, finally stained with substrate 3, 3'-diaminobenzidine.

ELISA

The recombinant protein expressed by pMYNS3 was purified by ion-exchange chromatography, and the purified protein was used as antigen to test 94 control positive sera by ELISA.

RESULTS

The PCR products were analyzed on 15g/L agrose gel electrophrosis, and the expected 634bp cDNA fragment was clearly seen. Sequencing results revealed that the cDNA fragment is 634bp, and proved to be HCV NS3 fragment in comparison of the sequence of cDNA fragment and the corresponding region of HCV-BK. Its nucleotide and amino acid homology with genotype II isolate HCV-BK, genotype I isolate HCV1, genotype III isolate HCV-J6 and genotype IV isolate HCV-J8 were 90.8% and 95%, 80% and 94.9%, 73.2% and 88.2%, 73.5% and 86.7%, respectively[8-10]. These results indicated that the isolate in this study belongs to HCV-II. In addition, we also compared its homology with the sequence of corresponding region of HCV isolates derived from the patients who came from Hebei and Taiwan separately, the nucleotide and amino acid sequence homology were 90.4% and 95.4%, 90.8% and 93.8%, respectively[11,12]. SDS-PAGE analysis demonstrated that pRXNS3 plasmid expressed a protein with a molecular weight of about *M*r 23 800 which amounts to 14% of the total bacterial proteins (Figure 1). pMYNS3 plasmid expressed a molecular weight of about *M*r 22 000 protein, which covers 11% of the total bacterial proteins (Figure 1). Two

forms proteins strongly reacted with anti-HCV antibodies in the sera in western blotting test (Figure 2). Using the purified pMYNS3 expressed recombinant protein as an antigen to test 94 control anti-HCV positive sera, 85 (90.4%) of 94 were detected. Among the 94 sera samples, 28 (96.6%) of 29 derived from patients with known transfusion history were detectable. Other sera derived from patients diagnosed clinically as having acute, chronic hepatitis and cirrohosis, the detection rates were 83.3% (10/12) for acute hepatitis, 86.8% (33/38) for chronic hepatitis and 93.3% (14/15) for cirrhosis, respectively.

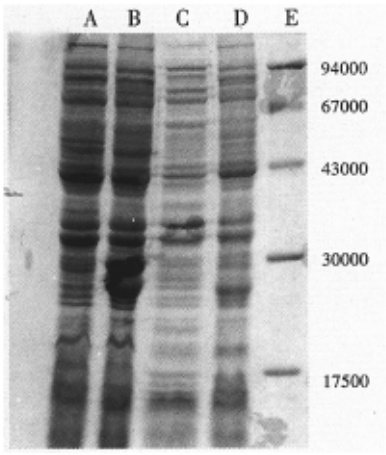

Figure 1 SDS-PAGE analysis of HCV NS3 fusion and non-fusion proteins expressed in *E. coli*
A: Control HB101/pRX B: Induced HB101/pRXNS3
C: Control HB101/pMY D: Induced HB101/pMYNS3
E: Molecular weight standards

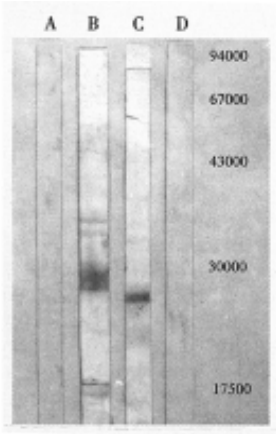

Figure 2 Western blot analysis of HCV NS3 recombinant proteins
A: Control HB101/pRX B: Induced HB101/pRXNS3
C: Induced HB101/pMYNS3 D: Control HB101/pMY

DISCUSSION

In this report, the NS3 fragment of HCV genome was reversed transcribed and amplified successfully from the serum of a patient who comes from Hebei Province, and the cDNA fragment was sequenced and expressed with high level in *E. coli*. The results of western blotting and ELISA showed greater antigenicity of the recombinant protein, which demonstrated good prospects of using the protein as an antigen to detect anti-HCV antibodies. The nucleotide and amino acid homology analysis indicated that the HCV isolate in this study should belong to HCV-II. Another HCV isolate derived from the same area is also HCV-II[11], which suggested in some extent that the endemic HCV isolates in this region might be HCV-II. The sequence comparison of NS3 region of HCV between different isolates showed that NS3 region is the relative conservative region in HCV genome, in addition, the amino acid homology is higher than nucleotide homology, so using NS3 protein as antigen to detect anti-HCV antibodies will be characterized by a wide coverage of HCV infection. The prokaryotic expression vector pRX and pMY were used to express HCV NS3 protein. pRX vector with trpE promotor is induced by 3-indolyl acrylic acid and expresses a fusion-protein with 18 amino acid at N-termini of the protein. pMY vector with p_Rp_L promoter is induced by heating at 42℃ and expresses non-fusion protein. We found that the expression level of fusion protein is higher than that of non-fusion protein. The possible explanation is that the proteinase of host bacteria will not recognize the recombinant fusion protein as non-self protein to lyse it. The fusion of bacterial protein to recombinant protein increases the stability of protein and raises the expression level. The comparison between the fusion and non-fusion protein in antigenicity showed no obvious difference. Because pMYNS3 is induced easily by heat, we used the protein expressed by pMYNS3 as coating antigen in ELISA. This study estimated the diagnostic value of NS3 recombinant antigen using 94 control anti-HCV positive sera which are detectable with Abbott second generation ELISA. The results showed that NS3 antigen can detect 85 (90.4%) of 94 of control sera, among these, 28 (96.6%) of 29 sera derived from hepatitis patients with known transfusion history were detected. These results consistent with the results of Abbott's ELISA test. The successful expression of HCV NS3 protein has provided an ideal basis for developing anti-HCV diagnostic reagents.

REFERENCES

1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*,1989;244(4902):359-362
2 Chomczynski P,Sacchi N.Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.*Anal Biochem*, 1987;162(1): 156-159
3 Rimm DL, Pollard TD. New plasmid vectors for high level synthesis of eukaryotic fusion proteins in Escherichia coli. *Gene*,1989;75(2):323-327
4 Takamizawa A, Mori C, Fuke I, Manabe S, Murahami S, Fujika J *et al*. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol*,1991;65(3):1105-1113ª¤
5 Widell A, Mansson AS, Sundstr-n G, Hansson BG, Nordenfelt E. Hepatitis C virus RNA in blood donor sera detected by the polymerase chain reaction: comparison with supplementary hepatitis C virus antibody assays. *J Med Virol*, 1991;35

(4):253-258
6 Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinstone M. Hepatitis C viral RNA in serum of with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primer sets. *Hepatology*, 1991;14(1): 51-55
7 Sambroo KJ, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed, New York: *Cold Spring Harbor Larbor Laboratory*, 1989:3-101
8 Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C et al. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA*, 1990;88(6):2451-2455
9 Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A *et al*. Nucleotide sequence of the hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J Gen Virol*, 1991;72(11):2697-2704
10 Okamoto H, Kurai K, Okada SI, Yamamoto K, Lizuka H, Tanaka T *et al*. Full length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology*, 1992;188(1): 331-341
11 Bi SL, Bai XH, Cong ME, Tian HW, Sun DG, Margolis HS et al. Primary structure and variation of Chinese hepatitis C virus genome. *Acta Virol*,1993;9 (2):114-127
12 Chen PJ, Lin MH, Tai KF, Liu PC, Lin CJ, Chen DS. The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. *Virology*, 1992;188(1):102-113

Combined Traditional and Modern Medicine

Short-term effect of Salvia miltiorrhiza in treating rat acetic acid chronic gastric ulcer and long-term effect in preventing recurrence

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Subject headings Danshen; disease models, animal; Salvia miltiorrhiza; stomach ulcer; acetic acid; cimitidine; gastric mucosa; recurrence; rats, Wistar; chronic diseases

Abstract

AIM To study the short-term effect of Danshen (Salvia miltiorrhiza) on acetic acid induced chronic gastric ulcer in rats and its long-term effect in preventing recurrence.

METHODS Rats with acetic acid-induced gastric ulcer were treated with Danshen and cimetidine for 30 days. Traditional gastric mucosal auto-radiography and ^{3}H-TdR incorporation into gastric mucosa in vitro were employed to study the effects of Danshen in rat acetic acid-induced chronic gastric ulcer, including ulcer index (UI), ulcer inhibitory rate (IR) and label rate (LR).

RESULTS On the day 5, 30 and 126 of ulcer-making, the UI in the Danshen group was obviously lower than that in the cimetidine group and the control group (42.3±3.9, 3.6±1.2, 4.4±2.3; 49.1±3.6, 5.9±1.4, 9.2±1.3; 61.0±3.8, 8.9±2.5, 12.4±2.4, respectively, $P<0.01$), the IR (%) in the Danshen group was obviously higher than that in the cimetidine group (31, 59, 64.8; 19, 33, 26, respectively), and the LR in the Danshen group was obviously higher than that in the cimetidine group and the control group (10.0±0.5,16.2±0.8, 15.0±0.6; 9.0±0.5, 13.9±0.6, 10.8±0.7; 6.5±0.7, 10.1±0.5, 8.0±0.7, respectively, $P<0.01$). There was no obvious difference in UI in the Danshen group on day 30 ascompared with that on day 126.

CONCLUSION Danshen is effective in promoting ulcer healing and preventing recurrence. The mechanism of action is to strengthen the gastric mucosal barrier and to promote the gastric mucosal cell proliferation along the edge of the ulcer.

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INTRODUCTION

Human peptic ulcer is characterized by repeated relapses and difficulty in prevention[1]. So, the emphasis of treatment of peptic ulcer should be put on preventing relapse. A rat model of acetic acid-induced chronic gastric ulcer has the features of repeated relapses and is similar to that of the human case[2,3]. This study is to observe the effect of the traditional Chinese medicine Danshen (Salvia miltiorrhiza) on the rat chronic gastric ulcer induced by acetic acid and its effect in preventing recurrence, and the relationship between the therapeutic effect and regeneration of gastric mucosal cells on the edge of the ulcer.

MATERIALS AND METHODS

Materials

Danshen water solution: prepared according to the reported method (240 g/L and 120 g/L)[4]. Cimetidine water solution: with cimetidine for injection (product of the 3rd Pharmaceutical Plant, Harbin), diluted to 2.4g/L and 1.2g/L water solution. ^{3}H-TdR supplied by China Atomic Scientific Institute, with radiation concentration of 18.5GBq/L.

Methods

Rat model-making and grouping. Wistar male rats (n = 126), weighing 210g ± 30g, were given 200ml/L acetic acid 0.05ml to create the model of chronic gastric ulcer, and then were divided into 3 groups randomly: Control group: allowed to drink water (pH 6.5) freely, continuing to day 126. Cimetidine group: During day 1-5 after ulcer was made, allowed to drink 2.4g/L cimetidine water solution (pH 6.5) freely; allowed to drink 1.2g/L

cimetidine water solution freely on day 6-30; only water freely on day 31-126. Danshen Group: On day 1-5 after the ulcer is made, allowed to drink 240g/L Danshen water solution (pH 6.5) freely; 120g/L Danshen water solution freely on day 6-30; only water freely on day 31-126. Preliminary tests proved that each rat drank liquid on an average of about 35ml/d, without statistical difference.

Gastric mucosal auto-radiography. A 0.5 cm × 1cm tissue piece taken for the ulcer edge was put into 2ml 1640 culture fluid (pH 7.2-7.2) containing ^{3}H-TdR and 100ml/L beef serum, hatched in 37℃ ±0.5℃ CO_2 incubator for 1 hour, fixed for 3-6 hours in Carnoy liquid, and then embedded in paraffin, 5 μm slices were made for HE staining, coating H-4 emulsion in emulsion-coating box, and to expose for 14 days in 4℃ refrigerator, and to develope, fix and take the nucleus over 5 silver granules as label cells.

Index and calculation. Ulcer index (UI)-ulcer maximum length × maximum width; ulcer inhibitory rate (IR) (%) = (1 -experimental group UI mean/control group UI mean) × 100%; label rate (LR) (%)-(label cells number/300 multiplication area cells)×100%.

Statistical analysis

All values were given as $\bar{x}\pm s$. Paired Student's *t* test was used. *P* values <0.05 were considered significant.

RESULTS

Effect of Danshen in rat chronic gastric ulcers of different stages, UI and ulcer IR is shown in Table 1.

Table 1 Rat chronic gastric ulcers of different stages, UI and IR ($\bar{x}\pm s$) (%)

Groups	Day 5			Day 30			Day 126		
	n	UI(mm)^2	IR(%)	*n*	UI(mm)^2	IR(%)	*n*	UI(mm)^2	IR(%)
Control	8	61.0±3.8		8	8.9±2.5^f		11	12.4±2.4	
Cimetidine	10	49.1±3.6^b	19	10	5.9±1.4^{bf}	33	11	9.2±1.3^b	26
Danshen	11	42.3±3.9^d	31	10	3.6±1.2^d	59	12	4.4±2.3^d	64.8

^{b}*P*<0.01, *vs* control; ^{d}*P*<0.01, *vs* Cimetidine; ^{f}*P*<0.01, *vs* the same group on day 126.

Table 2 Rat chronic gastric ulcers of different stages, IR of gastric mucosal cells (n = 6, $\bar{x}\pm s$)(%)

Groups	Day 5	Day 30	Day 126
Control	6.5±0.6	10.1±0.5	8.0±0.7
Cimetidine	9.0±0.5^b	13.9±0.6^b	10.8±0.7^b
Danshen	10.0±0.5^d	16.2±0.8^d	15.0±0.6^d

^{b}*P*<0.01, *vs* control group, ^{d}*P*<0.01, *vs* cimetidine group.

Effect of Danshen in rat chronic gastric ulcers of different stages and gastric mucosal cell multiplication is shown in Table 2

DISCUSSION

It is reported in the literature[5,6] that the natural healing process of acetic acid-induced gastric ulcer in rats is as follows: On day 1-35 UI decreased rapidly which is considered as acute stage; UI decreased steadily and slowly on day 36-80, which is a slow healing stage; UI slightly increased as the recurrence stage; UI increased obviously on day 120-140, the recurrence rate being the highest. In this experiment, we treated the ulcer with Danshen and cimetidine from day 1-30 to observe their therapeutic effects in the acute stage. During the period from day 31 to day 126 without the two drugs, we observed whether Danshen has a long-term effect in preventing ulcer recurrence. From Table 1, we can see the UI in the cimetidine group was obviously lower than in the control group (*P*<0.01), and that in the Danshen group was even lower than that in the cimetidine group (*P*<0.01). As for the ulcer IR Danshen group was definitely superior to the cimetidine group. This indicated that Danshen might promote ulcer healing. In comparison of the UI of each group on day 30 and day 126, significant difference was found in both the control and cimetidine groups (*P*<0.01), but in the Danshen group there was no significant difference on day 30 and day 126. This indicated that Danshen has significant effect in preventing recurrence. From Table 2, one could see a higher LR in the cimetidine group than in the control group (*P*<0.01), and the Danshen group had a higher rate than the cimetidine group (*P*<0.01). So, the mechanism of Danshen in promoting ulcer healing and preventing ulcer recurrence was an enhancement of gastric mucosal cell multiplication along the edge of ulcer and also a fortification of the mucosal barrier.

REFERENCES

1 Tao J. On some problems of peptic ulcer.*Jpn Med Introd*, 1992;8(13): 386-387
2 Zhang SH, Chen L, Chen DM, Shi AR.A method of making rat acetic acidulcer model.*Dissectol J*,1987;10(2):158-159
3 Houteng YM, Sangyun KZ. Experiment gastric ulcer model. *Clin Science*, 1990;26(1):87-94
4 Li HQ, Chang LG, Ju BQ, Liu RD. The study of the experiment that Danshen resists ulcer action.*J China Med Uni*,1985;4(2):89-92
5 Umeda Y, Nakamura K. Effects of Trimprostil on healing and recurrence of acetic acid-induced gastric ulcer in rats.*Jpn J Pharmacol*, 1986;42(8):457-463
6 Baimu ZY, Shanqi SY, Shisan GX, Shenbian MS, Shunei YY, Qiantian XE *et al*. Recurrence and relapse of acetic acid ulcer as well as effect of strengthening defence factor medicine Proamipide.*Folai Fharmacol Jpn*, 1988;92(6):389-395

Sishengtang decoction in alleviation of toxic and side effects of transarterial embolization *

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Subject headings Sishengtang decoction; liver neoplasms/therapy; embolization, therapeutic; immunity, cellular; killer cell, natural; killer cell, lymphokine-activated; leucocytes

Abstract

AIM To observe the therapeutic effects of Sishengtang decoction in alleviating the toxic and side effects of transarterial embolization (TAE).

METHODS Fifty-four patients with liver cancer were divided randomly into Sishengtang decoction group (34 cases) and control group (20 cases). The changes of clinical symptoms and peripheral hemogram and some cellular immune functions were observed before and two weeks after TAE.

RESULTS Sishengtang decoction was superior to the control group in improving the digestive tract reaction. The leucocytes of peripheral blood and cellular immune functions (activities of NK cells and LAK cells) of control group decreased obviously after TAE, while that of Sishengtang decoction group decreased slightly, without obvious difference as compared with that of preoperation.

CONCLUSIONS Sishengtang decoction might improve the clinical symptoms and increase the leucocytes of peripheral blood and the cellular immune functions of TAE patients.

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INTRODUCTION

After clinical and experimental studies of antiradioactive reaction produced by Sishengtang decoction[1], we studied systematically the therapeutic effects of this decoction in alleviating the toxic and side effects of chemotherapy, and observe the changes of peripheral hemogram and the cellular immune functions.

MATERIALS AND METHODS

General data

Of 54 patients with liver cancer, submitted for TAE from May 1995 to October 1996, 48 were males and 6 females, aged from 31 to 74 years, averaging 48.8 years. Twenty-one cases were in stage III and 33 were in stage III clinically. These patients were divided into Sishengtang decoction group (34 cases) and control group (20 cases). There were 29 males and 5 females in the Sishengtang decoction group, with an average of 49.2 years of age, and 14 cases in stage II and 20 in stage III. In the control group, there were 19 males and 1 female, averaging 48.1 years in age, 7 in stage II and 13 in stage III.

Component of the decoction

Sishengtang decoction contains mainly: *Radix Astragli sen Hedysari* 30g, *Radix Rehmanniae* 10g, *Rhizoma Atractylodis Macrocephalac* 9g and *Somen Colcis* 15g. Components were regulated according to symptoms (RCAS): *Radix Asparagi* 15g and *Radix Dphipogonis* 15g were added for mouth dryness; *Radix Codonopsis Pilosnlae* 30g and *Poria* 15g were added for lassitude; *Rhizoma Dioscoreae* 9g, *Endothelinm Cornenm Gigeriaw Galli* 12g, *Fructus Crataegi* 12g, *Fructus Hordei Germinatus* 12g and *Massa Fermentata Medicinalis* 12g were added for anorexia.

Administration and dosage

Sishengtang decoction and RCAS were used concomitantly in the Sishengtang decoction group. They were given a week before TAE, at 1 dose daily divided in the morning and evening. Only RCAS was used in the control group, administration and dosage were the same as the Sishengtang decoction group.

Observation

The changes in clinical symptoms of all the 54 patients were recorded one day and 5 days respectively after TAE and the peripheral hemogram and some

cellular immune functions were measured 1 week before and 2 weeks after TAE[2]. The activities of NK and LAK cells were detected by radioimmunoassay, with K_{502} and Raji, as target cells. Detailed processes were in accordance with Reference 2 and 3.

Statistical treatment

The data were expressed as $\bar{x}\pm s$, and the comparison between the two groups was made by χ^2 test and *t* test.

RESULTS

Changes in clinical symptoms

In 3 main clinical symptoms observed, the effect of Sishengtang decoction was superior to that of the control group (Table 1).

Changes in laboratory indexes

There was no obvious difference in peripheral hemogram and some cellular immune functions between Sishengtang decoction group and control group before therapy, but with no obvious decline of these two indexes in the control group after therapy (Table 2).

DISCUSSION

TAE is one of the most effective therapies for the patients who can not be operated on and for some of the patients with recurrent liver cancer after operation. But the toxic and side effects of TAE, such as decreased leucocytes in peripheral blood, reaction of digestive tract and decline of body immune functions, not only result in great pain for patients, but also become a main factor that influences the therapeutic effects of TAE. So it has been an important topic to seek effective traditional Chinese medicine or complex prescriptions which can reduce the toxic and side effects of chemotherapy.

Sishengtang decoction is a basic prescription, made according to the theory of traditional Chinese medicine and clinical experience from the specialists to relieve the patients from the toxic and side effects of radiotherapy and chemotherapy. In this prescription, *Radix Astragali sen Hedysari* invigorates the vital energy and the spleen; *Radix Rehmanniae* nourishes *yin* and invigorates the Kidney; *Rhizoma Atractylodis Macrocephalae* and *Semen Coicis* invigorate the spleen and regulate the middle warmer, and prevent the greasy of *Radix Astragali sen Hedysari* and *Radix Rehmanniae*. This prescription acts to invigorate both vital energy and *yin*, take care of Spleen and Kidney, not only prevent the exhaustion of congenital *yin* and *yang*, but also protect the acquired essential substance from food. Four elements of this prescription are all used unpreparedly in order to prevent too much dryness or greasiness of the herbal characters, which is of great significance in maintaining the process of digestion of the middle warmer and relieving the digestive tract reaction. According to the modern pharmacological analysis, *Radix Astragli sen Hedysari* and *Rhizoma Atractylodis Macrocephalac* can improve body immune function and increase leucocytes in peripheral blood; and *Somen Colcis* has the anticancer effects if used unprepared. When used together, these four elements will have the functions to reduce toxicin and promote their effects.

Table 1 Effects of Sishengtang decoction and RCAS on clinical symptoms of TAE patients ($\bar{x}\pm s$)

Groups	*n*	Significant improvement	Improvement	No improvement	Improvement rate(%)
Sishengtang decoction(34)					
Lassitue	28	14	12	2	93.9
Poor appetite	23	12	9	2	91.3
Nausea	23	11	9	3	86.9
Control (20)					
Lassitue	17	6	6	5	70.1[a]
Poor appetite	12	5	3	4	66.7[a]
Nausea	14	4	7	3	78.9[a]

[a]$P<0.05$ *vs* Sishengtang decoction

Table 2 Laboratory indexes of TAE patients in Sishengtang decoction group and control group($\bar{x}\pm s$)

Groups	*n*	WBC($\times10^9$/L)	PLT($\times10^9$/L)	NK activities(%)	LAK activities(%)
Sishengtang decoction	34				
Before treatment		4.62±0.62	123.00±37.45	32.03±7.25	34.07±6.69
After treatment		4.44±0.63	116.23±29.42	31.04±6.92	32.03±6.76
Control	20				
Before treatment		5.31±0.82	31.92±47.93	141.20±5.22	32.87±4.85
After treatment		3.87±0.38	126.80±37.58	26.16±7.77[a]	27.16±5.74[a]

[a]$P<0.05$ *vs* before treatment in control

In the improvement of clinical symptoms and results of laboratory examinations, RCAS, used in the control group, though can partially improve the clinical symptoms, its effects are not as good as Sishengtang decoction. As for the increase of leucocytes in peripheral blood, the effects of Sishengtang decoction group is also superior to that of the control group. The results indicated that Sishengtang decoction can alleviate the toxic and side effects of chemotherapy.

In term of the cellular immune functions of TAE patients, the activities of NK and LAK cells of the control group were decreased markedly, but without significant difference in the Sishengtang group compared with that of preoperation. The results showed that Sishengtang decoction might increase the leucocytes in peripheral blood and cellular immune functions, which is important in promoting the recovery of patients and enhancing the effects of TAE itself. The mechanism may be related to its enhancement and protection of blood production by marrow[4].

REFERENCES

1 Ling CQ, Chen LQ, Chen Z, Huang XQ. Clinical and experimental study of the resistance effects of Sishengtang decoction on toxic and side effects in radiotherapy and chemotherapy.*J Tradit Chin Med*, 1993;34(12):733
2 Chao XT. Basic and clinical study of Interleukin-2. *Beijing: Beijing Science and Technology Publisher*,1990:48-53
3 Zhu ZY. Methodology of practical medical tests. *Beijing: People's Military Medical Publisher*,1992:828-829
4 Ling CQ, Shi JH, sdsdsd Z, Zhang HW. The experimental study of Sishengtang decoction in promoting and protecting marrow blood making. *China J Basic Med Tradit Chin Med*,1997;5(2):68

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Reviews

DNA methylation and carcinogenesis in digestive neoplasms

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Subject headings DNA methylation; mutation; DNA methyltransferase; genes, suppressor tumor; digestive system neoplasms; p53 gene; gene expression

The DNA of vertebrates contains tissue specific patterns of methylated cytosine residues. These methylation patterns are transmitted by clonal inheritance[1] through the strong preferance of mammalian DNA (cytosine-5) methyltransferase (DNA-MTase) for hemimethylated DNA[2], and are established during early embryogenesis and gametogenesis[3]. Newly replicated DNA lacks this methylation in the nascent strand. Shortly after the passage of the replication fork, a maintenance DNA-MTase methylates CpG dinucleotides on the newly synthesized strand, thereby recreating the spectrum of methyl groups that existed prior to replication. DNA methylation (5-methylcytosine[5-mCyt] content of DNA) plays a considerable role in both normal development and carcinogenesis[4], but there seems to be conflicting reports concerning the role of DNA methylation in carcinogenesis. It has been hypothesized that hypomethylation of DNA facilitates aberrant gene expression in tumorigenesis[5]. Some believe that hypermethylation of DNA leads to the causative alteration in tumorigenesis which involves inactivating tumor suppressor genes and marking chromosome regions for deletion[6]. Some overlooked the importance of alterations in gene expression and thought that mutation played the key role[4]. All these help to achieve a better understanding of the mechanisms underlying carcinogenesis. We believe that carcinogenesis is a multistep/multistage process that occurs in animals[7] with more carcinogenesis than mutagenesis, and that DNA methylation played multiple roles in the transformation from a normal cell into a frank malignancy.

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DNA METHYLATION AND CANCER

Mutations which occur at CpG dinucleotides in vertebrate DNA can be attributed to the hydrolytic deamination of 5-mCyt and are easily recognized because of the nature of base substitution. Deamination of 5-mCyt dinucleotides results in the formation of thyminephosphoguanosine (TpG). This still does not account for the high frequency of mutagenesis observed at CpG sites. Differences in the repairing efficiencies of promutagenic lesions may be partly responsible for this discrepancy. The guanosine-thymine (G-T) misrepairs resulting from deamination of 5-mCyt are believed to be more difficult for the cell to repair, as thymine is a normal component of DNA.

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, esophagus and liver. Transitions predominate in colon, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the liver. Mutations at adeninethymine (A:T) base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. Most transitions in colorectal carcinomas are at CpG dinucleotide mutational hot spots. G-T transversions in esophageal carcinomas are dispersed among numerous codons[8]. One copy of the short arm of chromosome 17, which harbors the p53 gene, is lost in many human tumors including those of the colon and rectum. In the colon carcinomas 75% - 80% show a loss of both p53 alleles, one through deletion, the other through a point mutation. The point mutations are usually misense, giving rise to an altered protein[9]. Cytosine-thymine (C-T) transitions at CpG sites are the most prevalent mutations found in the p53 tumor suppressor gene in human colon tumors and in the germline (Li-Fraumeni syndrome). All of the mutational hot spots are methylated to 5-mCyt, and it has been hypothesized that the majority of these mutations are caused by spontaneous hydrolytic deamination of this base to thymine[10]. It is estimated that 75%-90% of hepatocellular carcinoma (HCC) cases are attributable to hepatitis B virus. HBV DNA integrates into HCC cells at random sites in the genome. It contains the X gene, which codes for a protein HBX that modu-

lates the transactivation of many cellular genes and is a candidate viral oncoprotein. HBX protein binds with p53 in vitro and in vivo[11], inhibits p53 sequence-specific DNA binding and transactivation activities, partially disrupts p53 oligomerization and prevents p53 binding to transcription-repair coupling factor ERCC3[12]. HBV infection alone does not influence the rate of p53 mutation, and aflatoxin B1 (AFB1) exposure is the most important factor influencing mutation prevalence. AFB1 plays an etiological role in HCC carcinogenesis and indicates a synergy between HBV and AFB1[13]. In patients with liver tumors from geographical areas where both AFB1 and HBV are cancer risk factors, most mutations are at one nucleotide pair of codon 249[8]. Multiple gene-tic alterations occur in gastric carcinomas, including point mutation of the *ras* oncogene and the p53 tumor suppressor gene, amplification of the c-met, k-sam, and c-erbB-2/neu genes, and loss of heterozygosity (LOH) of the bcl-2, APC, and DCC genes. Genetic changes in the transforming growth factor beta (TGF-β) type II receptor gene or altered expression of its messenger RNA[14] occur commonly in gastric cancer cells resistant to growth inhibitory action of TGF-β. Inactivation of the p53 gene through mutations and the allelic deletion may play an important role in gastric tumorigenesis. These mutations may cause a conformational change in the p53 protein, resulting in the loss of p53 suppression of the gastric cell growth partly through disruption of the association of p53 protein with a cellular component[15]. Allelic loss and mutation of the p53 gene are detected in over 60% of gastric cancers regardless of the histological type. Several gastric cancer cell lines have shown p53 gene abnormalities[16]. The c-met gene encoding the receptor for hepatocyte growth factor (HGF) is often amplified in advanced gastric cancer, particularly in 39% of scirrhous carcinomas, whereas c-met amplification is extremely rare in esophageal and colorectal cancers[17]. The interaction of c-met overexpressed tumor cells and HGF from activated stromal cells is involved in the morphogenesis and progression of gastric cancer. The K-sam gene encoding receptor for keratinocyte growth factor is amplified preferentially in the poorly differentiated scirrhous cancer[18]. However, no K-sam amplification is seen in esophageal and colorectal cancers. In general, K-sam amplification is independent of c-met amplification in scirrhous carcinoma, and c-erbB-2 gene amplification is detected only in well differentiated gastric cancer. Moreover, overexpression of c-erbB-2 is closely correlated with livermetastases. c-Ki-ras point mutation is observed in 9%-18% of well differentiated gastric cancers but not in poorly differentiated ones[19].

MECHANISMS OF DNA METHYLATION IN CARCINOGENESIS

Both general hypomethylation and regional hypermethylation coexist in the genome of a wide variety of human and animal cancers[5,6]. It has been suggested that changes in methylation may not play a causal role in carcinogenesis and could be consequences of the transformed state of tumor cells and that C-T transitions brought about by increased expression of the DNA methyltransferase play the key role[4]. Beside DNA MTase induced mutations additional factors appear to be involved in the cancer process. Cancers originate from a single cell that is changed dramatically by a series of alterations to the genome, e.g., mutation and changes in methylation altered gene expression. Mutagenesis plays a role in carcinogenesis. Mutated gene must be expressed to exert an effect.

It has been suggested that reduction of DNA MTase activity would lead to marked hypomethylation which can inhibit tumorigenesis[4,20]. Since DNA methylation is critical in development and differentiation[21] of tumors, it is reasonable to propose that hypomethylation at an intermediate level plays a key role in carcinogenesis while excessive hypomethylation may not be compatible with the life of the affected cells (e.g., owing to massive deregulation of gene expression). By this we mean that initiated cells may die under the severe conditions of hypomethylation and would not be available to form tumors. Thus the fact that inhibition of methylation may decrease tumor formation does not prove that excessive DNA MTase activity is the sole mechanism underlying carcinogenesis.

There are mechanistically and theoretically plausible genotoxic mechanisms that support the roles of both hypomethylation and hypermethylation of DNA (i.e. epigenetic changes) in carcinogenesis. These involve mainly alterations in normal gene expression (including tumor suppressor genes). Altered DNA methylation not only affects gene expression but also facilitates mutations, as 5-mCyt can deaminate spontaneously to Thymine (T). This indicates that elevated expression of the DNA MTase may lead to increased 5-mCyt, and this can increase the probability of C to T transitions[4,22,23]. Moderate DNA-MTase increases are not merely bystander effects, but could rather constitute one of the earliest and fundamental changes of neoplastic development[23]. Hypomethylation of DNA is associated with increased gene expression[24]. Additionally, a decreased capacity or fidelity of maintaining the normal methylation status of DNA may underlie the sensitivity of some mouse strains to liver tumorigenesis, in which increased expression of oncogenes appears to be involved by facilitating tumor promotion[5,25,27]. The

principal characteristic of the promotion stage of the carcinogenesis that distinguishes it from the stages of initiation and progression is its operational reversibility, i.e., clones of initiated cells regress when the promoting agent is withdrawn. The promotion stage ends when a lesion attains the capacity for growth in the absence of a promotion stimulus, i.e., when it is no longer reversible and can progress to a frank malignancy[7]. Hypermethylation of specific regions of DNA has been identified in cancer cells. For example, the Von Hippel Landau (VHL) tumor suppressor gene is hypermethylated and inactivated in a fraction of renal cell lines and tumors that did not have mutations in the coding regions sequenced[6]. Regional chromosomal hypermethylation is related to areas believed to contain tumor suppressor genes at various target sites. This regional hypermethylation in portions of the genome normally unmethylated may inactivate tumor suppressor genes. The functional significance would be the same as an inactivating mutation or as the loss of an allele. Regional hypermethylation of the retinoblastoma gene appears to inhibit transcription of this tumor suppressor genes[26]. Hypermethylation of the 5'CpG island of CDKN2/p16/MTS (tumor suppressor gene located on chromosome 9p21) is frequent in cell lines and primary lung tumors, gliomas and head and neck squamous cell carcinomas. Furthermore, inactivation through DNA methylation can occur not only in neoplasms where deletion is frequent in cell lines (breast, renal cells) but also in those which are not commonly associated with loss of p16 through homozygous deletion (colon and prostate). Hypermethylation of the p16 gene promoter region is a common abnormality of p16 gene in human cancer[27]. Nearly all acute leukemias and 50% of chronic leukemias and lymphomas show extensive methylation of the estrogen receptor (ER) gene CpG island[28]. Mutation in the p53 tumor suppressor gene is believed to occur in more than half of all solid tumors[29]. Approximately 24% of point mutations of p53 in human cancers are C-T transitions at CpG dinucleotides[29]. All of the mutational hot spots in the p53 gene occurring at CpG are methylated[30-32], suggesting an involvement of 5-mCyt as an endogenous mutagen. It is important to not only determine which tumor suppressor genes lie in specific regions and their normal functions but also demonstrate a causative role, perhaps by reversing their inactivation. Altered DNA methylation leads to 5-mCyt which has intrinsic hypermutability as compared with C via deamination. This causes C-T transitions[22]. In the presence of low levels of S-adenosyl methionine, DNA-MTase may be able to form uracil leading to C-T transitions[22]. Therefore, the high rate of mutation at CpG dinucleotides may be due, in part, to DNA-MTase mediated deamination[4,23], and inhibition of DNA mismatch repair£Û23£Ý. Additionally, 5-mCyt may influence carcinogenesis via inhibition of DNA repair, leading to fixation of promutagenic lesions[5,25]. However, the high percentage of mutations at CpG sites can not be explained solely by the presence of 5-mCyt, as the methylation patterns of the p53 gene are tissue independent, suggesting that tissue specific methylation does not contribute to the different mutation patterns at CpG sites seen in tumors[32]. This supports the contention that the effect of DNA methylation alterations may vary in different tissues and in the genesis of different tumor types.

The complex nature of the methylation role in region of gene expression is illustrated by the insulin growth factor type II receptor (Igf2r) gene. Both hypomethylation of the 5' flanking region and methylation of a specific CpG site in an intron are required for the occurrence of expression. The latter appears to be an imprinting signal, and hypomethylation at this site may silence the gene even if the 5'flanking region remains hypomethylated[33].

The epigenetic properties of DNA methylation are heritable and unlike the mutagenic effects of 5-mCyt which do not involve alterations of the primary DNA sequence. Methylation of cytosine residues contained in CpG islands of certain genes has been inversely correlated with gene activity, but it is still unclear whether this methylation is actually responsible for different activity states of a gene or is merely the result of such changes. Methylation at a CpG islands may lead to decreased gene expression by a variety of mechanisms including disruption of local chromatin structure, inhibition of transcription factor DNA, or by recruitment of proteins which interact specifically with methylated sequences indirectly preventing transcription factor binding[5,34]. Increased methylation and heterochromatization of CpG islands have been proposed as a mechanism for silencing the expression of non-essential genes during the establishment of immortal cell lines.

CONCLUSION

The current literature provides a compelling basis for suggesting that mutations arising secondarily to deamination of 5-mCyt, C or both are an important source of critical point mutations. Mutation, altered gene expression, hypomethylation and hypermethylation may be all related to carcinogenesis which are not mutually exclusive. Hypomethylation, hypermethylation and mutations may be important based on different situations. The examination of DNA methylation status provides the potential to discover alterations in gene expression, cell proliferation, mutation, chromatin aberrations and inactivation/

deletion of tumor suppressor genes in multifaceted approach that fits the multistep process of carcinogenesis. This notion can be supported by the depiction of human colon carcinogenesis in which roles for hypomethylation of DNA, mutation and tumor suppressor gene inactivation are considered to be relevant to the ultimate tumor formation[5,25]. There is a need to address the functional significance of specific changes in methylation (e.g., how the binding of transcating factors to specific genes is affected by methylation), and changes in methylation that occur in target tissues prior to the appearance of frank malignancies. The overall goal should be an understanding of changes in methylation and how they facilitate movement of cells through the different stages of carcinogenesis. This can be accomplished by keeping in perspective the fact that cancer is a disease of the whole entity, and thus there is a need to focus, though not exclusively, on in vivo studies.

REFERENCES

1 Wigler M, Levy D, Perucho M. The somatic replication of DNA methylation. *Cell*,1981;24(1):33-40
2 Graunbaum Y, Cedar H, Razin A. Substrate and sequence specificity of a eukaryotic DNA methylase.*Nature*,1982;295(5850):620-622
3 Chaillet JR, Vogt TF, Beier DR, Leder P. Parental specific methylation of an imprinted transgene is established during gametogenesis and progressive changes during embryogenesis.*Cell*,1991;66(1):77-83
4 Laird PW, Grusby JL, Fazeli A, Dickson S, Edward JW, Li E et al.Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, 1995;81(2): 197-205
5 Count JL, Goodman JI. Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion.*Mol Carcinogen*,1994;11(1):185-188
6 Herman JG, Latif F, Weng YK, Lerman MI, Zbar B, Liu S et al. Silencing of the VHL tumor suppressor gene by DNA methylation in renal carcinoma.*Proc Natl Acad Sci USA*,1994;91(21):9700-9704
7 Pitot HC, Dragon YP. Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J*,1991;5(9):2280-2286
8 Hollestein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers.*Science*,1991;253(5015):49-53
9 Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature*, 1991;351(6326):453-456
10 Schumutte C, Yang AS, Nguyen TDT, Beart RW, Jones PA. Mechanisms for the involvement of DNA methylation in colon carcinogenesis. *Cancer Res*, 1996; 56(10):2375-2381
11 Feitelson MA, Zhu M, Duan LX, London WT. Hepatitis BX antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma.*Oncogene*,1993;8(5):1109-1117
12 Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence specific DNA binding transcriptional activity, and association with transcription factor ERCC3. *Proc Natl Acad Sci USA*, 1994;91(6):2230-2234
13 Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT *et al*. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma.*Lancet*, 1992;339(8799): 943-946
14 Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Robert AB et al. Genetic changes in the transforming growth factor beta type II receptor (TGF-β) gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-β. *Proc Natl Acad Sci USA*,1994;91(19):8772-8776
15 Matozoki T, Sakamoto C, Suzuki T, Matsuda K, Uchida T, Nakano O *et al*. p53 gene mutations in the human gastric cancer: wild type p53 but not mutant p53 suppresses growth of human gastric cancer cells.*Cancer Res*,1992;52(16):4335-4341
16 Tahara E, Sembo S, Tahara H. Molecular biological observations in gastric cancer.*Seminars in Oncology*,1996;23(3):307-315
17 Kuniyasu H, Yasu W, Kitadai Y, Yokozaki H, Ito H, Tahare E. Frequent amplification of thec-met gene in scirrhous type stomach cancer. *Biochem Biophys Res Commun*,1992;189(1):227-232
18 Katoh M,Terada M.Oncogenes and tumor suppressor genes. In: Nishi M, Ichikawa H,Nakajima T *et al*,eds. Gastric cancer. Tokyo: Springer-Verlag, 1993:196-208
19 Tahara E. Molecular mechanisms of stomach carcinogenesis. *J Cancer Res Clin Oncol*,1993;119(5):265-272
20 Macleod AR, Szyf M. Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis.*J Biol Chem*, 1995; 270(14):8037-8043
21 Li E, Beard C, Jaenisch R. Role for demethylation in genomic imprinting. *Nature*,1993;366(6453):362-365
22 Yang AS, Shen JC, Zingg JM, Mi S, Jones PS. Hha I and Hha II DNA methyltransferases bind DNA mismatches methylate uracil and block DNA repair. *Nucleic Acids Res*,1995;23(8):1380-1387
23 Wu J, Herman JG, Wilson G, Lee YR, ChiuYen RW, Mabry M *et al*. Expression of prokaryotic Hha I DNA methyltransferase is transforming and lethal to NIH 3T3 cells.*Cancer Res*,1996;56(3):616-622
24 Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer can reactivate estrogen receptor gene expression.*Cancer Res*,1995;55(11):2279-2283
25 Counts JL, Goodman JI. Molecular and cellular mechanisms. In: Liver regeneration and carcinogen.*New York: Academic Press*,1995:227-255
26 Greger V, Debus N, Lohmann D, Hopping W, Passage E, Horsthemke B. Frequency and parental origin of hypermethylated RBI alleles in retinoblastoma. *Hum Genet*,1994;94(5):491-496
27 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JPJ, Davidson NE. Inactivation of the CDKN2/16/MTS gene is frequently associated with aberrant DNA methylation in all common human cancers.*Cancer Res*,1995;55(20):4525-4530
28 Issa JPJ, Zehnbauer BA, Civin CI, Collector MI, Sharkis SJ, Davidson NE *et al*. The estrogen receptor CpG island is methylated in most haemopoetic neoplasms. *Cancer Res*,1996;56(5):973-977
29 Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer aetiology and molecular pathogenesis.*Cancer Res*,1994;54(18):4855-4878
30 Rideout WM, Coetzee GA, Olumi AF, Jones PA. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science*, 1990;249 (4974):1288-1290
31 Mague AN, Jones PA. Ubiquitous and tenacious methylation of the CpG site in codon 248 of the p53 gene may explain its frequent appearance as a mutation hot spot in human cancer.*Mol Cell Biol*,1994;14(6):4225-4232
32 Tornaletti S, Pfeifer GP. Complete and tissue independent methylation of CpG sites in the p53 gene: implications for mutations in human cancers. *Oncogene*, 1995;10(8):1493-1499
33 Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H et al. Maternal specific methylation of the imprinted mouse Igf 2r locus identifies the expressed locus as carrying the imprinting signal.*Cell*, 1993;73(1):61-71
34 Bird AP. The essentials of DNA methylation. *Cell*, 1992;70(1):5-8

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Progress in endoscopic management of pancreas diseases

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Endoscopy has been widely used in the treatment of many biliary tract discorders and as an alternative to many surgical procedures. The remarkable advances achieved in the performance of therapeutic endoscopic retrograde cholangio-pancreatography(ERCP) have placed endoscopy in the forefront for treating pancreatico-biliary diseases[1]. It has established itself as a valuable tool. However, it is only recently that these techniques have been applied and adopted to disorders of the pancreas for their frequent and potentially serious complications and technical problems, etc. Generally, therapeutic ERCP of the pancreas is still in its infancy.

Recently, endoscopic treatment, especially therapeutic ERCP for pancreas diseases has developed rapidly, such as acute pancreatitis, chronic pancreatitis, pancreatic pseudocysts or pancreas divisum. These will be reviewed as follows.

ACUTE PANCREATITIS

Acute gallstone pancreatitis

Gallstones are one of the major etiological factors of acute pancreatitis. 60% of the patients with acute pancreatitis are found to have gallstones, and 68% of the severe cases have[2,3] gallstones in common bile duct. Conservative medical management alone for gallstone pancreatitis fails in 20% - 30% of patients, with more complications and higher mortality. In 1974, Cotton and Beales showed that acute pancreatitis could not be considered contraindicated for ERCP[4]. Recently, urgent (within 24-72 hours) ERCP and endoscopic sphincterotomy ES have been performed in those patients with suspected acute gallstone pancreatitis. Urgent ERCP usually shows normal pancreatic duct, and being less helpful to the diagnosis of mild pancreatitis and surgical indications. However, it does not prolong hospital stays or increase morbidity. Controlled study showed that patients benefited from early ERCP and ES as demonstrated by lower morbidity (24% *vs* 64%), shorter hospitalization (9.5% days *vs* 17 days), and lower motality (2% *vs* 8%). No serious procedure-induced complications were observed in the studies[2]. It suggested that urgent ERCP and ES are safe and effective for patients with acute biliary pancreatitis.

Fan *et al*[5] reported their results with early ERCP and ES in 195 patients with suspected acute gallstone pancreatitis, among whom 127 (65%) had gallstones in common bile duct or gall bladder. Those who underwent ERCP had nobiliary sepsis, and the mortality was also low. Patients with a mild attack of pancreatitis should be closely observed and an immediate intervention is not- necessary. Urgent ERCP and ES should be reserved for patients with an attack of pancreatitis predicted to be severe, those whose pancreatitis does not subside with conservative measures over 72 hours.

Acute recurrent pancreatitis

Acute recurrent pancreatitis is defined as two or more attacks of pancreatitis associated with at least twice normal serum amylase levels[6]. Those patients are always without a positive history of alchol abuse or gallstone. ERCP can disclose an etiological factor such as bile duct stones, choledochocoles, papillary tumors, duodenal duplication cysts, intraductal strictures, pancreas divisum, or papillary stenosis in 40%-50% of cases. In about a half of those, ERCP can reveal a motor abnormality of Oddi sphincter (normal£¼30mmHg) or dysfunction of Oddi sphincter. The management of acute recurrent pancreatitis includes balloon dilatation of the stricture and endoprosthesis. If the recurrent attacks of pancreatitis relate closely to Oddi sphincter dysfunction, endoscopic sphincterotomy of pancreatic duct sphincter should be performed as a routine. This procedure can decrease the pressure of the pancreatic duct, and relieve symptoms of pancreatitis. At present, pancreatic sphinterotomy is not recommended for patients without sphincteric dysfunction and a normal pancreatic duct[2].

CHRONIC PANCREATITIS

Chronic pancreatitis is a disease that is difficult to manage. It has three types of clinical presentations, including abdominal pain (70% of cases may be related to the increased pancreatic duct pressure), diarrhea (20%-30%), endocrine failure in the form of diabetes (3%-37%). ERCP can reveal pancreatic duct stricture, stones in pancreatic duct or pan-

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creatic pseudocysts. So the endoscopic management includes pancreatic sphincterotomy, balloon dalatation of strictures, pancreatic stent and pseudocyst drainage.

To those patients with pancreatic duct stricture, a stiff nylon catheter or a Soehendrea dilating catheter is introduced over a guidewire to dilate the stricture up to 7-Fr. The catheter is then removed, and over the guidewire an endoprosthesis (5-or 7-Fr) is inserted. Most strictures will recur soon after dilation alone. Improvement of pain was seen in 54% - 90% of cases after pancreatic stenting. Pancreatic stenting might occlude side branches and subsequently cause formation of cystic lesion in the side branches. The stent can also induce inflammatory changes of the duct which mimics chronic pancreatitis[7]. There is a tendency to leave the endoprosthesis in place for as short a period as possible (about 6 w). Recurrence of abdominal pain often indicates stent blockage and the necessity to replace or remove the stent. Additional side flaps outside the papilla may prevent the stent form imigrating into the pancreatic duct[8]. The migrated stent can be extracted by endoscope immediately or surgical intervention if necessary. Endoscopic stenting is a useful alternative to surgical treatment and has lower morbidity and mortality. It does not impair the remaining pancreatic function, and surgery is still possible, if necessary.

It is not clear whether pancreatic stones are the cause or the results of recurrent attacks of pancreatitis. They can be extracted by balloon catheter or baskets. If stones can not be removed completely during the first endoscopic session, a stent can be inserted beyond the stones for adequate drainage until the second attempt. Larger stones above a stricture can befragmented within the pancreatic duct by using extracorporal shock wave lithotripsy (ESWL). In series of 123 patients, stones were fragmented successfully in 99% cases with the main pancreatic duct being completely cleared in 59%. After a mean follow-up of 14 months, complete or partial pain-relief could be obtained for 85% of the patients[12]. Electromagnetic lithotripsy (EML) can also be applied. Smith reported 15 cases, and complete clearance of pancreatic duct was seen in 60% of patients.

PANCREATIC PSEUDOCYSTS OR PANCREATIC ABSCESSES[2,9-11]

Patients with pancreatic pseudocysts have clinical features as chronic pancreatitis, they also have obstruction of biliary and upper gatrointestinal tract. Endoscopic treatment includes nasopancreatic drainage, cystogastrostomy or cystoduodenostomy and endoprosthesis with lower morbidity and mortality in comparing with surgical procedures. Chronic pseudocysts more than 4cm-6cm should be drained soon after the diagnosis was made. Nasopancreatic drainage is always used to perform systogastrostomy or cystoduodenostomy if the pseudocyst is closely in contact with the gastric or duodenal wall, as evidenced by CT or endosonography. Endoscopic cystoduodenostomy (ECD) is the first choice for treatment of paraduodenal cysts, whereas endoscopic cystogastrostomy (ECG) is an alternative procedure for the drainage of retrogastric cysts. Binmoeller *et al* reported their results of 53 patients who had undergone transpapillary drainage of ECD and/or ECG, cysts disappeared in 95% of them. Cremer *et al* reported 33 cases, and no recurrence of pseudocysts or complications was seen. Symptoms were relieved completely in 90% of the patients.

Endoscopic drainage is also safe and effective in pancreatic abscesses. Binmoeller et al reported 10 cases. Abscesses completely resolved in 8 patients. During a mean follow-up of 10 months, none of the patients had recurrence.

PANCREAS DIVISUM

Pancreas divisum is a congenital abnormality found in 5%-7% of patients[6]. Most investigators believe that the relatively small size of the minor papilla, which drains the largest part of the pancreas, could cause pancreatic congestion, leading to pain or pancreatitis. Endoscopic sphincterotomy of the minor papilla and endoprosthesis are always performed, and symptoms improve significantly in 83%-90% of cases.

Endoscopic sphincterotomy of the minor papilla can enlarge outflow of the pancreatic duct, but stricture recurs quickly and causes attacks of pancreatitis, so a stent (5- or 7-Fr) is always inserted after the sphincterotomy. Lehman *et al* reported that patients with recurrent pancreatitis benefited from endoscopic therapy more often than those with chronic pancreatitis or chronic pain during the mean follow-up of 1.7 years[12]. In patients with pancreas divisum and pancreatitis, stenting of the minor papilla was associated with a 49% complication rate, and was always moderate or severe. Endoscopic stent was not effective but associated with 80% of complications in those cases with pain only and a normal ERCP[13].

The long-term success of stent treatment is unknown. Stenting may cause dilation of the minor papilla sphincter. The results of this study suggest that impairment of pancreatic drainage by the minor papilla is probably an etiological factor for recurrent pancreatitis in patients with pancreas divisum.

PERIPHERIC TUMORS OF VATER'S AMPULLA

ERCP is mainly used for the diagnosis of peripheric tumors of Vater's ampulla. Endoscopic appearance, radiography, forceps biopsy, collection of pancreatic cells and pancreatic secretion can afford evidences of pathology and photology. To those benign tumors such as adenomas and malignant ones that

cannot be removed, complete removal and conservative medical treatment should be performed, respectively. Binmoeller *et al*[14] reported 25 cases of removal of benign adenomas with standard polypectomy snare, all were successful. Its criteria were: size less than 4 cm; and benign histologic findings on forceps biopsy (minimum of six biopsies). It is dangerous with more complications, so its indications are those who refused to undergo surgical treatment and those at high surgical risk.

COMPLICATIONS

The complications of endoscopic management of pancreatic disorders are much higher than those of biliary tract diseases (about 25%).

The common complications of endoscopic treatment are pancreatitis, hemorrhage, perforation and infection. Abdominal discomfort and sepsis are frequent in the management of endoscopic sphincterotomy and stone extraction. Complications of cystogastrostomy and cystoduodenostomy are mainly hemorrhage and perforation, and technique-related infection, for example, the infection of cysts. Stent dysfunction, inducing clogging migration of the stent and erosion of the opposite wall of the duodenum, appear to be frequent and major longtermproblem. Stent clogging is reduced through self-expandable metal stents, but experience in the pancreatic duct is limited and these stents are not easy to be removed. Most complications of endoscopic the rapy can be successfully treated conservatively or endoscopically, and surgical procedure is also needed if necessary.

In summary, selection of patients for the various treatment options should be strict and is probably important for optimum results of therapy because of their technique and complications, especially in children[15].

REFERENCES

1 Siegel JH, Cohen SA. Therapeutic pancreaticobiliaty endoscopy. *Gastroenterology*,1995;3(1):28-40
2 Huibregtse K, Smits ME. Endoscopic management of disease of the pancreas. *Am J Gastroenter*, 1994;89(8):66-77
3 Carr-Locke DL. Role of endoscopy in gallstone pancreatitis. *Am J Surg*,1993; 165(4):519-520
4 Cotton PJ, Beales JSM. Endoscopic pancreatography in the management of acute pancreatitis.*Br Med J*, 1974;1(5):608-611
5 Fan ST, Lai ECS, Mok EPT, Lo CM, Zheng SS, Wong J et al.Early treatment of acute biliary pancreatitis by endoscopic papillotomy. *N Engl J Med*,1993;328 (2):228-232
6 Bedford RA, Howerton OH, Geenen JE. The current role of ERCP in the management of benign pancreatic disease.*Endoscopy*, 1994;26(2):113-119
7 Sherman S, Alverez C, Roberts M, Ashlev S, Reber H, Lehman G *et al*.Polythylene pancreatic duct (PD) stent induced changes in the normal dog pancreas. *Gastrointest Endosc*,1993;39(2):A332
8 Johanson J, Schmalz M, Geenen JE. Simple modification of a pancreatic duct stenting to prevent proximal migration.*Gastrointest Endosc*, 1993;39(1): 62-64
9 Binmoeller KF, Walter A, Seifert H, Sochendra N. Endoscopic stenting for pancreatic pseudocysts in 53 patients.*Gastrointest Endosc*,1993;39(2):A240
10 Binmoeller KF, Walter A, Seifert H, Soehendra N. Endoscopic therapy for pancreatic abscesses. *Gastrointest Endosc*,1993;39(2):A239
11 Howell DA, Mnggia RA, Bosco JJ, Biber BP. Transpapillary pancreatic duct endoprosthesis in the management of pancreatic pseudocysts. *Gastrointest Endosc*, 1993;39(2):A317
12 Lehman GA, Sherman S, Nisi R, Hawes RH. Pancreas divisum: results of minor papilla sphincterotomy.*Gastrointest Endosc*,1993;39(1):1-8
13 Rosch J, Allescher HD. Update in gastroenterologic endoscopy: a review of endoscopy (abstracts presented at 1993 DDW in Boston).*Endoscopy*,1993;25(4): 401-422
14 Binmoeller KF, Boaveture S, Ramsperger K, Soehendra N. Endoscopic snare excision of benign adenoma of the papilla of Vater.*Gastrointest Endosc*,1993;39 (2):127-131
15 Brown KO, Goldschmiedt M. Endoscopic therapy of biliary and pancreatic disorders in children.*Endoscopy*, 1994;26(9):719-723

Brief Reports

Combined measurement of serum tumor markers in patients with hepatocellular carcinoma *

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Subject headings liver neoplasms/diagnosis; carcinoma, hepatocellular/diagnosis; tumor markers, biological/blood; alpha-fetoproteins/blood; sialic acid/blood; fucosidase/blood; enzyme linked immunosorbent assay; spectrophotometry

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms, and its prognosis is very poor if diagnosed late, therefore, early detection is important. As we know not all HCC can secrete AFP, and AFP levels may be normal in as many as 40% of patients with early HCC and 15%-20% of patients with advanced HCC[1]. Therefore, we selected AFP, alpha-L-fucosidase (AFU) and sialic acid (SA) in combination for detecting HCC.

MATERIALS AND METHODS

Subjects were divided into three groups: ① HCC group consisted of 30 patients (26 males and 4 females) with a mean age of 50.4±12.7 years; ② liver cirrhosis group consisted of 30 patients (24 males and 6 females) with a mean age of 45.3±8.4 years; and ③control group consisted of 30 healthy subjects (28 males and 2 females) with a mean age of 33.2±4.8 years whose liver function tests were normal. Diagnosis of HCC was in accordance with the criteria of the National HCC Association of China in 1977[2].

The fasting sera from all subjects were stored at -18℃. AFP was measured by ELISA. The kits were provided by Xiamen Advanced Scientific Institute[3]. AFU was measured according to Troost's method and expressed as nkat/L[3]. The kits were provided by Sanming Lanbo Biological Technique Institute. SA was measured by spectrophotometry. The kits were purchased from Dongou Biological Technical Institute. The data were expressed as $\bar{x}\pm s$ and analyzed statistically by the Student's *t* test.

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RESULTS

The serum levels of AFP, AFU and SA in the three groups are shown in Table 1.

The serum levels of AFP, AFU and SA in patients with HCC were significantly higher than those in patients with cirrhosis ($P<0.01$) and in the control subjects ($P<0.01$). No significant differences were found between the latter two groups.

The cutoff value was defined as $\bar{x}+2s$ (AFP≤30 μg/L, AFU≤180 nkat/L and SA≤630 mg/L).

The positive rates and significance of AFP, AFU and SA in the three groups are shown in Tables 2 and 3.

Results of combined measurement with two positives among the three tumor markers are shown in Table 4.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate were 86.7%, 98.3%, 93.7%, 96.3% and 94.4%, respectively.

Table 1 The serum levels of AFP, AFU and SA among patients in the three groups (x±s)

Groups	AFP(μg/L)	AFU(nkat/L)	SA(mg/L)
Control	10.2±9.8	106.0±36.5	513.7±57.8
Cirrhosis	14.4±9.0	126.8±52.1	522.7±70.5
HCC	71.7±38.8[bd]	284.5±102.6[bd]	636.7±76.6[bd]

[b]$P<0.01$, compared with controls, [d]$P<0.01$, compared with cirrhosis.

Table 2 Positive rates of AFP, AFU and SA among the three groups

Groups	AFU		AFU		SA	
	n	(%)	*n*	(%)	*n*	(%)
Controls	1	(3.3)	1	(3.3)	2	(6.7)
Cirrhosis	2	(6.7)	4	(13.3)	5	(16.7)
HCC	21	(70.0)	23	(76.7)	21	(70.0)

Table 3 Significance of AFP, AFU and SA (%)

	AFP	AFU	SA
Sensitivity	70.0(21/30)	76.7(23/30)	70.0(21/30)
Specificity	95.0(57/60)	91.7(55/60)	88.3(53/60)
Positive predictive value	87.5(21/24)	82.1(23/28)	75.0(21/28)
Negative predictive value	86.4(57/66)	88.7(55/62)	85.5(53/62)
Accuracy	86.7(78/90)	86.7(78/90)	82.2(74/90)

Table 4 Results of combined measurement of serum AFP, AFU and SA in the three groups

Groups	Postivity	
	n	%
Controls	0	0.0
Cirrhosis	1	3.3
HCC	26	86.7

Patients with HCC showed both positive AFU and AFP in 16/30 patients and negative results in 2/30 patients. Of the remaining patients, 5 were positive in AFP and negative in AFU, and 7 were negative in AFP and positive in AFU (Table 5). No correlation was found between AFP and AFU ($P<0.05$).

Table 5 Comparison between AFP and AFU in patients with HCC

AFP	AFU		
	Positive	Negative	Total
Positive	16	5	21
Negative	7	2	9
Total	23	7	30

DISCUSSION

So far, AFP still remained the most sensitive and specific marker of HCC. Our results showed that the sensitivity of AFP in patients with HCC was 70%, concordant with the reports by many other authors[4,5].

AFU is a lysosomal enzyme involved in the catabolism of the fucose-containing glycoconjugates. In accordance with the study of Deugnier, et al, the serumlevel of AFU activity in patients with HCC was increased[6]. Its positive rate in our study was 76.7% (23/30), similar to other reports (75%-76.7%)[4-7]. This may be related to increased enzyme release by tumor cells[6,7].

Our study showed that serum AFP and AFU activity was independent with no correlations between them as shown in Table 5. Therefore, AFU may be considered as an additional useful marker for detection of HCC.

The SA was considered to be associated with behaviour of malignant tumors. In our studies, its positive rate in patients with HCC was 70% as reported by many other authors[8,9]. The serum level of SA in HCC was also high and was positive in 71.4% when AFP was negative.

The main purpose of using combined measurement of tumor markers is to eliminate the false negative and false positive results as reported by many authors[1,4,5,10]. We used combined measurement of serum AFP, AFU and SA of patients with HCC. With this measurement, any two positives among the three tumor markers as the diagnostic criteria, the sensitivity, specificity, positive predictive value, negative predictive value and rate of accuracy in HCC patients were 86.7%, 98.3%, 93.7%, 96.3% and 94.4%, respectively. Six of 9 patients with HCC who had negative AFP had positive results in both AFU and SA. None of 3 patients with liver cirrhosis and controls who had positive AFP had positive results in AFU and/or SA. Therefore, combined measurement of serum AFP, AFU and SA is of practical significance in diagnosis of HCC.

REFERENCES

1 Giardna MG, Matarazzo M, Varriale A, Morante R, Napoli A, Martino R. Serum alpha L fucosidase: a useful marker in the diagnosis of hepatocellular carcinoma.*Cancer*,1992;70(5):1044-1048
2 Editorial Board of Practical Oncology. Practical oncology.*Beijing: People's Health Publisher*,1979:112-142
3 Troost J, Van der Heijden M, Staal G. Characterization of alpha-L-fucosidase from two different families with focosidosis.*Clin Chim Acta*, 1976;73(2):329-346
4 He M, Shi YR, Zou W. Combined assay of serum AFU and AFP in patients with primary hepatic carcinoma.*Chin J Clin Oncol*,1996;23(1):21-23
5 Shen W, Shen DM. Preliminary study of serum α-L-fucosidase assay in diagnosis of hepatocellular carcinoma.*Chin J Intern Med*, 1989;28(7):397-399
6 Deugnier Y, David V, Brissot P, Mabo P, Delamaire D, Messner M et al. Serum α-L-fucosidase: a new marker for diagnosis of primary hepatic carcinoma. *Hepatology*, 1984;4(5):889-892
7 Zhang SM, Wu MC, Chen H, Tu ZX, Cui ZF, Yu ZX *et al*. Value of serum glycosidase spectrum in the diagnosis of hepatocellular carcinoma. *Chin J Oncol*, 1992;14(2):143-145
8 Liu JL, Cai ZD, Liu RT, Zhang QS, Zhang YK, Che LH *et al*. The significance of serum sialic acid in the diagnosis of primary hepatic carcinoma. *J Pract Internal Med*,1991;11(11):576-577
9 Pan J, Geng JM, Zou JX, Wen GY. Improved colorimetry assay of serum sialic acid in the patients with primary hepatic carcinoma. *J Clin Lab Sci*,1991;9(3):124-125
10 Tu ZX, Yin ZF, Cui ZF, Wu MC. Prediction of primary liver cancer with alpha-fetoprotein. *Acad J Sec Mil Med Univ*,1991;12(5):417-419

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Effect of various drinking water on human micronucleus frequency in high risk population of PHC

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INTRODUCTION

The relationship between PHC and contamination of drinking water has been reported in some papers [1,2]. In Qidong, a PHC high-incidence area, the epidemiological investigation showed that there may be one or various carcinogens in the drinking water from various sources [3], but these data were epidemiological results. Using a simple and effective micronucleus testing technique to evaluate environmental contamination, we observed the micronucleus effect of human peripheral lymphocytes induced by the drinking water from various sources. We reviewed and analyzed previous research data, together with the results of epidemiological investigation, in order to provide more convincing experimental evidence for the pathogenesis of PHC induced by drinking water.

MATERIAL AND METHODS

Experimental materials

Water samples. Water samples were taken from house pool water (HPW), field canal water (FCW) and tap water (TW or deep well water). Water samples of these 3 types were collected in the same area, filtered, boiled and concentrated 100 times for use. Double distilled water (DDW) was used as the control. Blood samples. Obtained from 38 year old male volunteers with no diseases in physical check up.

Experimental methods

Preparation of micronucleus. Put 0.3ml venous blood from volunteers into each culture flask containing heparin 50mg/L, and then add PHA 0.2ml, RPMI-1640 culture solution (contains 20% bovine serum) 4.5ml, and water samples 0.05ml-0.15ml. Keep the flask at 37℃ in an incubator for 72 hours, and shake them every 12 hours. Micronucleus was prepared according to the method reported by us [4].

Indices observed. ① Cell growth of each experimental group each day, and ② micronucleus. Using a double blind test, we checked each sample under oil immersion objective to choose transformed lymphocytes which are intact and clear. Micronucleus number was counted with ‰ in 2000 lymphocytes of each blood sample. The standard to evaluate micronucleus has been reported [5].

Experimental grouping

Each of the 3 experimental groups (HPW, FCW and TW) was divided into 3 subgroups according to different doses of water samples (0.05ml, 0.10ml and 0.15ml) together with DDW, there were totally 10 experimental groups.

RESULTS

Morphologic observation of cultural cells in each experimental group

The cytotoxicity of 3 subgroups of HPW was very strong, with only a few transformed lymphocytes, and damaged membrane structure of many cells, so that the micronuclei could not be observed and the micronucleus frequency (MNF) could not be counted. The cell growth was in relatively good condition in FCW. The majority of lymphocytes were transformed with a full appearance and had intact membrane, but the cytotoxicity was enhanced with increasing doses of experimental water, and numbers of micronuclei induced were also increased. The situation of cell growth in TW groups was better than that of FCW groups. There was a weak cytotoxicity, MNF in each subgroup was lower than that of FCW groups. The cell growth condition of DDW groups was the best. There were lots of clear, transformed lymphocytes with intact membrane, abundant cytoplasm and few micronuclei.

Dose-response relationship of MNF induced by water in each experimental group

Compared with DDW group, the MNF induced by FCW groups was the highest, and that of TW water groups was relatively lower, which rose with increasing doses of water in each experimental group (Figure 1).

Figure 1(PDF) Dose-response relationship of MNF induced by various water samples.

DISCUSSION

Primary hepatocellular carcinoma (PHC) is one of ten common malignant tumors in the world. Qidong is a high-risk area of PHC in China, and its annual mortality remains at above 50/100000. Genetic epidemiological and experimental research data showed that PHC was caused by a complex of etiologic factors, including HBV infection, liver disease history, heredity, sex, etc [6,7]. Contamination of drinking water may be one of the causes of PHC. The results of this study showed that the degree of cytotoxicity of drinking water from various sources was in the order of HPW＞FCW＞TW＞DDW and the effect of various drinking water samples on inducing micronuclei of human peripheral lymphocytes was FCW＞TW＞DDW, and MNF was elavated with the increasing dose of each experimental group. Previous research data showed that the incidence rates of PHC varied in Qidong residents who drank water from various sources. The incidence was HPW (141.40/100000), FCW (72.32/100000), river water (43.45/100000), shallow well water (22.26/100000) and deep well water (11.70/100000) [3],

with significant difference ($P<0.01$) . This suggests that MNF induced by drinking water from different sources correlates with the incidence of PHC in the population who drink the corresponding water. In other words, if the residents drink badly polluted water, the effect on micronucleus will be strong, and incidence rate of PHC will be high. Our experimental results are consistent with the results of epidemiological investigation. These data have confirmed the close relationship between drinking water and PHC incidence in the high-risk population of PHC, and the polluted drinking water may be one of the causal factors in PHC.

Recent reports showed that microcystin contents (MC), a kind of PHC carcinogen from a certain poisonous algae, was detected in the drinking water of Haimen area, a neighbour of Qidong City. The positive rates (>50ng/L) of MC in drinking water from various sources were 17.3% (house pool water), 31.9% (river water), 4.3% (shallow well water) and 0.0% (deep well water). The average contents of each of these water samples were 101ng/L, 160ng/L, 68ng/L and 0ng/L, respectively. The difference was significant ($P<0.01$) [8] . The more serious the contamination of drinking water, the higher is its MC content. The relationship between MC and PHC incidence rate in residents drinking water from various sources should be further studied. It can be suggested that drinking deep well water, or improving the quality of drinking water may be of great significance in the prevention of PHC.

REFERENCES

1 Su DL. Drinking water and liver cancer. Chin J Prev Med, 1980;14(2):65-68

2 Zhang LS, Yang KZ, Hou JL, Meng GR, Huang ZY, Wu JL. The effect of micronucleus on contaminative water in the high morbid area of liver cancer and study on carcinogenic potentiality.Cancer,1988;7(1):1-4

3 Shen ZC, Li WG, Chen JG, Xie JR. The epidemiological research on relationship between various drinking water types and incidence of PHC. Jiangsu Med J,1985;11(1):28-31

4 Liu E, Zhang QN. The simple slide-making method of micronucleus of lymphocytes cultured in vitro.J Clin Lab Sci, 1992;10(2):105

5 Zhang QN, Li WG, Liu E, Huang QS, Yu SY, Zhu YJ. The effect of Se-intaking on micronucleus frequency of peripheral lymphocytes in HBsAg positive patients who were induced by AFB-1.Cancer Res Prev Treat,1993;20(1):3

6 Shen FM, Le MK, Cong HM, Wang JM. Complex segregation analysis of primary hepatocellular carcinoma in Chinese families: interaction of inherited susceptibi-lity and hepatitis B viral infection.Am J Hum Genet, 1991;49(2):88-92

7 Liu E, Wu Y, Zhang BC. Epidemiologic and clinical features of primary hepatocellular carcinoma.Jiangsu Med J, 1993;19(5):247-248

8 Chen G, Yu SZ, Wei GR, Chen GC, Xu XY, Huang YH et al. Studies on microcystin contents in different drinking water in highly endemic area of liver cancer.Chin J Prev Med,1996;30(1):6-9